Glycogen formation in *Corynebacterium glutamicum* and role of ADP-glucose pyrophosphorylase

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Glycogen is generally assumed to serve as a major reserve polysaccharide in bacteria. In this work, glycogen accumulation in the amino acid producer *Corynebacterium glutamicum* was characterized, expression of the *C. glutamicum glgC* gene, encoding the key enzyme in glycogen synthesis, ADP-glucose (ADP-Glc) pyrophosphorylase, was analysed, and the relevance of this enzyme for growth, survival, amino acid production and osmoprotection was investigated. *C. glutamicum* cells grown in medium containing the glycolytic substrates glucose, sucrose or fructose showed rapid glycogen accumulation (up to 90 mg per g dry weight) in the early exponential growth phase and degradation of the polymer when the sugar became limiting. In contrast, no glycogen was detected in cells grown on the gluconeogenic substrates acetate or lactate. In accordance with these results, the specific activity of ADP-Glc pyrophosphorylase was 20-fold higher in glucose-grown than in acetate- or lactate-grown cells. Expression analysis suggested that this carbon-source-dependent regulation might be only partly due to transcriptional control of the *glgC* gene. Inactivation of the chromosomal *glgC* gene led to the absence of ADP-Glc pyrophosphorylase activity, to a complete loss of intracellular glycogen in all media tested and to a distinct lag phase when the cells were inoculated in minimal medium containing 750 mM sodium chloride. However, the growth of *C. glutamicum*, its survival in the stationary phase and its glutamate and lysine production were not affected by *glgC* inactivation under either condition tested. These results indicate that intracellular glycogen formation is not essential for growth and survival of and amino acid production by *C. glutamicum* and that ADP-Glc pyrophosphorylase activity might be advantageous for fast adaptation of *C. glutamicum* to hyperosmotic stress.

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INTRODUCTION

Glycogen is a polysaccharide consisting of glucose units in a branched structure, comprising α -1,4 glucosyl linkages and a smaller number of α -1,6 branching linkages. In most bacteria, glycogen accumulates in the stationary growth phase and under conditions of limited growth in the presence of an excess of carbon and energy (Preiss & Romeo, 1994). Therefore, glycogen is generally assumed to be a storage compound serving as a carbon and energy reserve (reviewed by Preiss, 1984, 1996). Consistent with this assumption, some bacterial mutants deficient in glycogen synthesis show decreased survival under starvation conditions, when compared to the wild-type (Preiss, 1984). However, glycogen obviously is not essential for bacterial growth since many of these mutants grow as well as their parental strains (Preiss, 1984). In some bacteria, such as

Bacillus subtilis and *Streptomyces coelicolor*, glycogen may play a role in sporulation and differentiation, respectively (Kiel *et al.*, 1994; Martin *et al.*, 1997), and in *Mycobacterium smegmatis*, glycogen has been proposed to serve as a carbon capacitor for glycolysis during exponential growth, in addition to its conventional storage role (Belanger & Hatfull, 1999). Furthermore, glycogen biosynthesis recently has been connected with osmotically regulated trehalose synthesis in *Corynebacterium glutamicum* (Tzvetkov *et al.*, 2003; Wolf *et al.*, 2003; Padilla *et al.*, 2004b). This organism forms trehalose as a compatible solute under certain (hyper)osmotic stress conditions and additionally as an essential cell wall compound (Wolf *et al.*, 2003; Puech *et al.*, 2000; Argüelles, 2000; Daffé, 2005). In contrast to other bacteria, the osmotically induced trehalose synthesis in *C. glutamicum* has been shown to be mainly mediated by the TreYZ pathway (Wolf *et al.*, 2003). This pathway uses (poly)maltodextrins, such as glycogen, as a substrate and thus, glycogen formation in *C. glutamicum* might be involved in osmoprotection of this organism.

Abbreviations: ADP-Glc, ADP-glucose; CAT, chloramphenicol acetyltransferase; INT, iodinitrotetrazolium chloride; WT, wild-type.

The pathway of glycogen biosynthesis in bacteria proceeds by the action of three enzymes (Fig. 1), i.e. ADP-glucose (ADP-Glc) pyrophosphorylase (ATP: α -D-glucose-1-phosphate adenylyltransferase; EC 2.7.7.27), glycogen synthase (EC 2.4.1.21) and branching enzyme (EC 2.4.1.18), encoded by the *glgC*, *glgA* and *glgB* genes, respectively (Preiss, 1984, 1996). The key regulatory step of this pathway is the reaction of ADP-Glc pyrophosphorylase, which forms ADP-Glc and pyrophosphate from ATP and α -D-glucose 1-phosphate. Most ADP-Glc pyrophosphorylases are allosterically controlled (positively and negatively) by various intermediates of the major carbon and energy metabolic pathways (Ballicora *et al.*, 2003; Preiss, 1996, 1984). The genetic aspects of glycogen biosynthesis have been studied most comprehensively in *Escherichia coli* (reviewed by Preiss, 1996). In this organism the genes encoding the glycogen biosynthetic enzymes are organized in a cluster which includes the *glgC*, *glgA* and *glgB* genes and two genes involved in glycogen degradation, *glgX* and *glgY* (Romeo *et al.*, 1998). The *glgY* gene is now designated *glgP* (Preiss, 1996) and encodes a glycogen phosphorylase (EC 2.4.1.1) which catalyses glycogen breakdown by removing glucose units from the nonreducing ends (Alonso-Casajús *et al.*, 2006), whereas the *glgX* gene recently has been shown to encode an isoamylase-type debranching enzyme (EC 3.2.1-) with high specificity for hydrolysis of polysaccharide outer chains that have been recessed previously by glycogen phosphorylase (Dauvillée *et al.*, 2005). The *E. coli glg* gene cluster is composed of two tandemly arranged operons (*glgCAP* and *glgBX*) and the transcription of these operons is subject to complex regulation including catabolite repression, stringent control and/or control by RpoS, KatF, GlgQ, GlgR and/or the carbon storage regulator CsrA (Baker *et al.*, 2002; Romeo & Preiss, 1989; Yang *et al.*, 1996; Preiss, 1996). Clustering of genes involved in glycogen metabolism has been also described for other bacteria, e.g. *Bacillus stearothermophilus* and *B. subtilis* (Takata *et al.*, 1997; Kiel *et al.*, 1994), *Rhodobacter sphaeroides* and *R. capsulatus*

(Igarashi & Meyer, 2000), *Mesorhizobium loti* (Lepek *et al.*, 2002) and *Agrobacterium tumefaciens* (Ugalde *et al.*, 1998), although the precise genetic organization and transcriptional regulation obviously differs among these organisms.

C. glutamicum is an aerobic, Gram-positive organism that grows on a variety of sugars and organic acids and is widely used in the industrial production of amino acids, particularly L-glutamate and L-lysine (Eggeling & Bott, 2005). The genome of this organism has recently been determined and annotated (GenBank accession nos NC_003450 and BX927147; Kalinowski *et al.*, 2003; Ikeda & Nakagawa, 2003) and open reading frames putatively encoding several enzymes involved in glycogen metabolism were detected (Kalinowski *et al.*, 2003). These genes include *glgC* (*cg1269*), *glgA* (*cg1268*), *glgB* (*cg1381*), *glgE* (*cg1382*), two *glgP* genes (*cg1479* and *cg2289*) and a *glgX* gene (*cg2310*), the latter four possibly encoding a glucanase, two glycogen phosphorylases and a debranching enzyme, respectively. However, so far none of the respective enzyme activities have been proven to be present in *C. glutamicum* and, with the exception of *glgA*, involvement of the genes and their products in glycogen synthesis or degradation has not been shown. In the course of studies on trehalose biosynthesis, Tzvetkov *et al.* (2003) recently found that inactivation of the chromosomal *glgA* gene led to the failure of sucrose-grown *C. glutamicum* cells to accumulate glycogen. Thus, although there is evidence for the presence of glycogen in *C. glutamicum*, knowledge of glycogen formation and degradation, the enzymes involved, their regulation, and the function of glycogen in *C. glutamicum* is meagre. In this communication we report on the glycogen content of *C. glutamicum* during growth in different media, its ADP-Glc pyrophosphorylase activity, the analysis, expression and inactivation of the *glgC* gene, and the significance of glycogen synthesis for amino acid production and for osmoprotection of *C. glutamicum*.

METHODS

Bacterial strains, plasmids and culture conditions. The strains used in this study were *E. coli* DH5 α (Hanahan, 1983), *C. glutamicum* wild-type (WT; ATCC 13032 from the American Type Culture Collection) and its lysine-producing derivative *C. glutamicum* DM1730 (*pyc*^{P458S} *hom*^{V59A} *lysC*^{T311I} *zwf*^{A243T}) (Georgi *et al.*, 2005; obtained from B. Bathe, Degussa AG, Halle-Künsebeck Germany). Plasmid pK19*mobsacB* (Schäfer *et al.*, 1994) was employed for construction of the *glgC* mutants *C. glutamicum* WT-IMC and DM1730-IMC; plasmid pET2 (Vášicová *et al.*, 1998) was used for transcriptional fusion experiments.

E. coli and all pre-cultures of *C. glutamicum* were grown aerobically in TY or LB complex medium (Sambrook & Russell, 2001) at 37 °C and 30 °C, respectively. For the main cultures of *C. glutamicum*, the cells of an overnight pre-culture were washed with 0.9 % (w/v) NaCl and inoculated into TY medium with or without 1 % (w/v) glucose or into minimal medium (Eikmanns *et al.*, 1991) containing glucose, fructose, sucrose, acetate or lactate at concentrations indicated in the Results and Discussion section. *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 (see

Fig. 1. Schematic diagram of glycogen synthesis in *E. coli*. Gene names for the respective enzymes are given in italics.

below). In standard glutamate fermentations, ethambutol (25 mg l⁻¹) was added to the culture 2 h after inoculation, as described by Radmacher *et al.* (2005). The growth of *E. coli* and of *C. glutamicum* was monitored by measuring the OD₆₀₀.

Parallel fermentations with *C. glutamicum* were performed at 30 °C in 450 ml glass fermenters ('Fedbatch-pro system' from DASGIP). The pH was maintained at 7.0 using a standard pH electrode (Broadley James Corporation) and addition of 2 M KOH and/or 2 M HCl. Foam development was controlled by manual injection of small amounts (about 100 µl) of silicon antifoam (Roth). The dissolved oxygen was measured online using a polarimetric oxygen electrode (Broadley James Corporation) and it was adjusted to 30 % of saturation during constant aeration with 2 vvm [30 l h⁻¹] in a cascade by stirring at 150 to 1200 r.p.m.

Competition experiments between *C. glutamicum* WT and *C. glutamicum* WT-IMC were performed as follows. The OD₆₀₀ of both pre-cultures was determined; the proportions of both pre-cultures in the common inoculum to give an OD₆₀₀ of 1 in the main culture were calculated according to the ratios of *C. glutamicum* WT and *C. glutamicum* WT-IMC stated in the Results and Discussion section. The ratio of *C. glutamicum* WT and *C. glutamicum* WT-IMC in the culture broth during the competition experiments was monitored by live-cell counting on LB plates with and without kanamycin (25 µg ml⁻¹).

DNA preparation, transformation and DNA manipulations. Standard procedures were employed for plasmid isolation, for molecular cloning and transformation of *E. coli* DH5 α , and for electrophoresis (Sambrook & Russell, 2001). *C. glutamicum* chromosomal DNA was isolated according to Eikmanns *et al.* (1994). Plasmid DNA from *E. coli* was isolated using the method of Birnboim (1983). *C. glutamicum* WT and DM1730 were transformed by electroporation using the method of Tauch *et al.* (2002). The resulting transformants were selected on LB-BHIS agar plates containing kanamycin (25 µg ml⁻¹). All restriction enzymes, T4 DNA ligase, calf intestine phosphatase and *Taq* polymerase were obtained from MBI-Fermentas or from Roche Diagnostics and used according to the instructions of the manufacturer.

RNA techniques. Total RNA from *C. glutamicum* was isolated and purified as described by Schreiner *et al.* (2005). For Northern (RNA) hybridization, a digoxigenin-dUTP-labelled *glgC*-specific 0.5 kb DNA probe was generated by PCR with primers IM-*glgC*-for (5'-ACG-CGTCGACACCACGTGTATCGCATGG-3') and IM-*glgC*-rev (5'-CGGGATCCTGTGGATTGGCCACTCAG-3'; *Bam*HI restriction site underlined). For hybridization, total RNA from *C. glutamicum* WT was separated on an agarose gel containing 17 % (v/v) formaldehyde and transferred onto a nylon membrane (Eikmanns *et al.*, 1994). Hybridization [at 50 °C, in the presence of 50 % (v/v) formamide], washing and detection were carried out using the Nucleic Acid Detection kit (Roche Diagnostics). The size marker was the 0.24–9.5 kb RNA ladder from GibcoBRL.

Cloning of the *glgC* promoter. The *glgC* promoter fragment was amplified from chromosomal DNA of *C. glutamicum* WT by PCR with the primers PRAC-2-for (5'-ACGCGTCGACTGCACCCATGCAGTGAAC-3'; *Sall* site underlined) and PR-*glgC*-rev (5'-CGGGATCCGAATCCGAGTTCACCAAG-3'; *Bam*HI site underlined). The 437 bp PCR product, covering the region from 263 bp upstream to 174 bp downstream of the translational start codon, was digested with *Sall* and *Bam*HI, ligated into *Sall*/*Bam*HI-restricted plasmid pET2 and transformed into *E. coli*. The recombinant plasmid pET-PC was then isolated from *E. coli* and introduced into *C. glutamicum* by electroporation. The nucleotide sequence of the promoter fragment in plasmid pET-PC was verified by sequence analysis (MWG Biotech).

Construction of the *glgC* mutants of *C. glutamicum*. The chromosomal *glgC* gene in *C. glutamicum* was disrupted by the method described by Schäfer *et al.* (1994). For this purpose, a 0.5 kb internal fragment of *glgC* was amplified by PCR with primers IM-*glgC*-for and IM-*glgC*-rev (see above), restricted with *Sall* and *Bam*HI, and the resulting 0.37 kb DNA fragment was inserted into plasmid pK19mobsacB, which is nonreplicative in *C. glutamicum*. The resulting plasmid pK19IMC was transformed into *C. glutamicum* WT and DM1730; transformants were obtained by selection on medium containing kanamycin (15 µg ml⁻¹). Kanamycin resistance indicated integration of pK19IMC into the chromosomal *glgC* gene via recombination. To confirm integration, we performed a Southern blot analysis, essentially as described by Reinscheid *et al.* (1999). *Bam*HI-restricted chromosomal DNA from *C. glutamicum* WT, and from putative *glgC* integration mutants *C. glutamicum* WT-IMC and DM1730-IMC, was hybridized to a digoxigenin-dUTP-labelled 0.37 kb internal *glgC* probe (prepared from plasmid pK19IMC), resulting in two signals, at 9.2 kb and 7.1 kb, with the DNAs from *C. glutamicum* WT-IMC and DM1730-IMC and one signal, at 10.2 kb, with DNA from *C. glutamicum* WT. These sizes were expected for the *glgC* integration mutants and the WT strain, respectively.

Analysis of intracellular glycogen. The glycogen content of *C. glutamicum* cells was determined either enzymically (Parrou & Francois, 1997) or by TLC essentially as described by Seibold *et al.* (2006). For the enzymic assays, 5 ml samples of the culture were harvested and the cells were washed twice with TN buffer (50 mM Tris, 50 mM NaCl; pH 6.3) and once with resuspension buffer (40 mM potassium acetate; pH 4.2). The cells then were suspended in resuspension buffer (total volume 1 ml) and transferred to 2 ml screw-top Eppendorf tubes filled with 250 mg acid-washed glass beads (150–212 µm; Sigma-Aldrich). After inactivation of cell-bound glycosidic activity by incubation at 95 °C for 5 min, the cells were disrupted using a Ribolyser (Hybaid) three times without cooling at maximum speed of 6.5 for 45 s. The cell debris and glass beads were separated from the supernatant by centrifugation (13 000 g, 20 min). Each sample was divided into two 100 µl aliquots (assays A and B), and 2 µl amyloglucosidase (10 mg ml⁻¹; Roche Diagnostics) was added to assay A; assay B was used as a reference. Both assays were incubated with shaking for 2 h at 57 °C. The glucose concentration was then measured in both control (assay B; without amyloglucosidase) and test (assay A; with amyloglucosidase) tubes as described below. The amount of glucose determined in the control assay B was subtracted from the amount of glucose determined in assay A. The cell dry weight of *C. glutamicum* was calculated according to the OD₆₀₀; an OD₆₀₀ of 1 corresponded to 0.25 g l⁻¹ for *C. glutamicum* (Börmann *et al.*, 1992).

TLC was used to distinguish between glycogen and other (lower molecular mass) carbohydrates that might serve as substrates for amyloglucosidase. Aliquots (10 ml) of the culture were withdrawn, and the cells were pelleted by centrifugation for 5 min at 13 000 g and washed twice with TN buffer following a washing step with water. The cell pellet was resuspended to a total volume of 1 ml with water and transferred into a 2 ml screw-top Eppendorf tube filled with 250 mg acid washed glass beads. Enzyme activity was inactivated, cells were disrupted and cell debris and glass beads were removed by centrifugation as described above. Sample preparation, application, separation and visualization of the carbohydrates on the TLC plate (by spraying with sulphuric acid solution and successive charring) were performed as previously described (Seibold *et al.*, 2006). Carbohydrates were identified by comparison to the migration of authentic standards (glucose, maltose, maltotriose, maltoheptaose and glycogen; all except glucose obtained from Sigma Aldrich).

Quantification of glucose and of amino acids. For analysis of the glucose or amino acid concentrations in the culture broth, 1 ml

of the culture was withdrawn and centrifuged (13 000 g, 10 min, 4 °C) and the supernatant was used for the determination. The amino acid concentrations were determined as described by Schrupf *et al.* (1991). Glucose was determined enzymically using hexokinase/glucose-6-phosphate dehydrogenase (Roche Diagnostics) and spectrophotometric quantification (at 340 nm) of the NADPH formed.

Enzyme assays. ADP-Glc pyrophosphorylase activity was determined by measuring the formation of glucose 1-phosphate from ADP-Glc and pyrophosphate similar to the method described by Takata *et al.* (1997). *C. glutamicum* cells were harvested, washed twice in 0.1 M Tris/HCl, pH 8, 20 mM KCl, 5 mM MgSO₄ and resuspended in 0.75 ml of the same buffer containing 0.1 mM EDTA, 2 mM DDT and 10% (v/v) glycerol. The cell suspension was transferred to 2 ml screw-cap vials together with 250 mg glass beads and subjected five times for 30 s to mechanical disruption with a Ribolyser at 4 °C, with intermittent cooling on ice for 5 min. After disruption, the glass beads and cellular debris were removed by centrifugation (10 000 g, 4 °C, 15 min). Afterwards the supernatant was centrifuged (45 000 g, 1.5 h) to remove the membrane fraction. Carbohydrates such as maltodextrins, serving as a substrate for background glucose 1-phosphate formation, were removed by use of a HiTrapDesalting column (Amersham Bioscience) equilibrated with 0.1 M Tris/HCl, pH 8, 25 mM MgCl₂ on an Äkta Purifier System (Amersham Bioscience). Up to 200 µl of the enzyme preparation was added to a total volume of 500 µl of reaction mixture containing 0.1 M Tris/HCl, pH 8, 25 mM MgCl₂, 2 mM sodium pyrophosphate, 1.5 mM iodinitrotetrazolium chloride (INT), 5 U phosphoglucomutase (from rabbit muscle; Roche Diagnostics), 5 U glucose-6-phosphate dehydrogenase (from *Leuconostoc* sp.; Roche Diagnostics) and 5 U diaphorase (from pig heart, Roche Diagnostics). The reaction was initiated at 30 °C by addition of 5 mM ADP-Glc and the change of absorption at 492 nm due to the reduction of INT was monitored in a spectrophotometer (Ultrospec 2100 pro, Amersham Bioscience) for 3 min. As a control, the change in absorption at 492 nm in the absence of ADP-glucose was measured and subtracted from the experimental values. One unit is defined as the amount of enzyme that produces 1 µmol glucose 1-phosphate in 1 min.

To determine chloramphenicol acetyltransferase (CAT) activity, crude extracts were prepared as described above, except that the cells were 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). The biuret method (Gornall *et al.*, 1949) with bovine serum albumin as the standard was used to determine protein concentrations.

Computational analysis. MFold (Zuker, 2003) software was used for the calculation of the ΔG° value (free energy under standard conditions) of the *glgC* terminator structure. The protein and DNA sequences were analysed using the ExPASy proteomics server (Gasteiger *et al.*, 2003) and CLUSTALW (Thompson *et al.*, 1994). The SWISSPROT accession number for *C. glutamicum* ADP-Glc pyrophosphorylase is Q8NRD4. Additional ADP-Glc pyrophosphorylase sequences used for comparison with this polypeptide and their respective accession numbers are as follows: *Mycobacterium tuberculosis* (P64241), *S. coelicolor* (P72394), *E. coli* (POA6V1) and *A. tumefaciens* (Q8V8L5).

RESULTS AND DISCUSSION

Growth and glycogen content of *C. glutamicum* in different media

The glycogen content of *C. glutamicum* WT cells was determined during growth in different complex and minimal

media. No glycogen was detected in the cells when *C. glutamicum* WT was cultivated in TY and LB complex media without an additional carbon or energy source. Addition of 1% or 2% (w/v) glucose to TY or LB medium led to the formation of up to 70 mg glycogen per g dry weight during exponential growth and subsequent degradation of the glycogen in the early stationary phase. In complex medium containing 4% (w/v) glucose, the cells accumulated up to 90 mg glycogen per g dry weight. For investigation of glycogen synthesis of *C. glutamicum* in minimal medium containing different carbon sources, we inoculated all following cultures with cells pre-grown in TY medium without additional glucose, i.e. with glycogen-less cells. The growth of *C. glutamicum* in minimal medium with 2% (w/v) glucose, 2% (w/v) fructose or 2% (w/v) sucrose as single carbon source was very similar, resulting in final optical densities (OD₆₀₀) of about 36.7, 34.5 and 36.5, respectively (Fig. 2a). With 5% (w/v) glucose, the cells grew to an OD₆₀₀ of about 60. Cells of all four cultures showed rapid glycogen accumulation in the early exponential growth phase and degradation of the polymer before they entered the stationary phase, i.e. when the carbon source became limiting. However, the maximal intracellular glycogen concentration varied in the cells grown on the different substrates (Fig. 2b). Whereas *C. glutamicum* in minimal medium with glucose or sucrose accumulated up to 90 mg glycogen per g dry weight, the cells cultured in minimal medium with fructose as single carbon source accumulated only about 40 mg glycogen per g dry weight. The lower glycogen accumulation on this substrate may be due to different entry points of the substrates into the central metabolism, i.e. glucose 6-phosphate (the direct precursor of glucose 1-phosphate) in the case of glucose and sucrose and fructose 1,6-phosphate (which has to be dephosphorylated and isomerized to glucose 6-phosphate) in the case of fructose (Yokota & Lindley, 2005). It should be noted that the maximal glycogen content of the *C. glutamicum* cells was about 35% lower when they were grown in media containing only 1% (w/v) glucose instead of 2%. Although increasing the initial glucose concentration to 5% (w/v) led to slightly higher glycogen concentrations in the early stationary phase (about 45 mg per g dry weight at after 9 h), it did not lead to higher intracellular glycogen concentrations or to a significantly different glycogen profile in the course of growth.

To examine whether glycogen is also formed during growth of *C. glutamicum* on substrates requiring gluconeogenesis, the organism was grown in minimal medium with either 1% (w/v) potassium acetate or 1% (w/v) sodium lactate. The growth of *C. glutamicum* in minimal medium with these carbon sources differed only slightly (growth rates of about 0.29 and 0.30 h⁻¹, respectively). The cells grown on acetate accumulated small amounts of glycogen (2–5 mg per g dry weight), whereas no glycogen was detected in the samples derived from cells grown in minimal medium containing lactate. Thus, *C. glutamicum* was shown to form glycogen when growing exponentially in media containing sugar

Fig. 2. Growth (a) and intracellular glycogen content (b) of *C. glutamicum* in minimal medium containing different sugars. Circles and white bars, 2% glucose; triangles and black bars, 2% fructose; squares and light grey bars, 2% sucrose; diamonds and dark grey bars, 5% glucose.

substrates and to degrade the glycogen formed as soon as, or even slightly before, entering the stationary phase when the carbohydrates become limiting.

ADP-Glc pyrophosphorylase activity in *C. glutamicum*

The specific activity of ADP-Glc pyrophosphorylase was determined in cell extracts of *C. glutamicum* WT grown in TY complex medium and in minimal medium containing glucose, potassium acetate or sodium lactate [all at 1% (w/v)] as the carbon source and harvested at the early exponential growth phase (3 h after inoculation). The highest specific activity [6.0 mU (mg protein)⁻¹] was found in cells grown in minimal medium containing glucose. The activity was about 20-fold lower when the cells were grown in TY complex medium or in minimal medium containing acetate or lactate [0.2–0.4 mU (mg protein)⁻¹]. The dependency of ADP-Glc pyrophosphorylase activity on the growth phase was elucidated by measuring the specific activity in extracts from *C. glutamicum* WT in minimal glucose medium harvested at the early, mid and late exponential phase and at the stationary phase. The highest specific activity was found when the cells were harvested in the early exponential phase [6.0 mU (mg protein)⁻¹]; slightly lower activities were found in cells at the mid and late exponential phase [5.2 mU (mg protein)⁻¹] and at the stationary phase [3.8 mU (mg protein)⁻¹]. These results suggest that ADP-Glc pyrophosphorylase activity in *C. glutamicum* is severely regulated by the carbon source in the growth medium and only very weakly regulated by the growth phase. However, it should be stated that the specific ADP-Glc pyrophosphorylase activity did not correlate with the cellular glycogen content observed in the course of growth. This result indicates that glycogen synthesis in *C. glutamicum* is controlled either by another biosynthetic

enzyme (e.g. glycogen synthase or branching enzyme) or by activity regulation of ADP-Glc pyrophosphorylase by (a) so far unidentified effector(s). Here it is noteworthy to mention that all known bacterial ADP-Glc pyrophosphorylases, with the exception of those in *B. stearothermophilus* and *B. subtilis*, are allosterically controlled by small effector molecules, such as metabolites of the glycolytic pathways (as activators) and AMP, ADP and inorganic phosphate (as inhibitors) (reviewed by Ballicora *et al.*, 2003). The probability of allosteric regulation of the *C. glutamicum* ADP-Glc pyrophosphorylase is strengthened by the identification of amino acid residues known to be important for the regulation of the corresponding *E. coli* enzyme (see below).

Analysis of the *glgC* gene and its chromosomal organization

The *C. glutamicum* *cg1269* gene (identical to NCgl1073) was sequenced in the course of the determination of the genome sequence (accession nos NC_003450 and BX927147) and has been designated ADP-Glc pyrophosphorylase gene *glgC* (Kalinowski *et al.*, 2003; Ikeda & Nakagawa, 2003). It has a length of 1218 bp and the predicted gene product (accession no. Q8NRD4) consists of 405 amino acids with a molecular mass of 43 861 Da. The *glgC* gene is preceded by a probable ribosome-binding site (AAGGG) and followed by a region of dyad symmetry similar to rho-independent transcription terminators [centred 18 bp downstream of the TAA stop codon; $\Delta G^{0'} = -14.7$ kcal mol⁻¹ (-61.5 kJ mol⁻¹) at 25 °C]. This result indicates transcriptional termination downstream of the *glgC* gene. The *glgC* gene is located upstream of and in the opposite orientation to *cg1268*, which putatively encodes a glycogen synthase and is therefore annotated as *glgA*. Tzvetkov *et al.* (2003) inactivated this gene in *C. glutamicum* and found the mutant to be unable to accumulate glycogen. Downstream

of, and also in the opposite orientation to *glgC*, there is a gene (*cg1270*) annotated as 'probable O-methyltransferase' (Kalinowski *et al.*, 2003). Except for a putative fructose hydrolase gene (*cg1267*) directly downstream of *glgA*, no other genes involved in glycogen or carbohydrate metabolism were identified near *glgC* and *glgA*. Putative *glgB* (*cg1381*), *glgP* (*cg1479* and *cg2289*) and *glgX* (*cg2310*) genes (encoding glycogen synthetic and degradation enzymes) have been identified elsewhere in the *C. glutamicum* genome (Kalinowski *et al.*, 2003) and thus, unlike the clustering of five *glg* genes (*glgC*, *glgA*, *glgB*, *glgP* and *glgX*) in other bacteria (Igarashi & Meyer, 2000; Preiss, 1996), the *C. glutamicum glg* genes (or their homologues) obviously are scattered within the genome.

Database analysis and alignment studies with the *C. glutamicum* protein encoded by *glgC* revealed significant similarity of the *C. glutamicum* protein to the functionally well-characterized ADP-Glc pyrophosphorylase from *E. coli* (Preiss & Romeo, 1994; 40 % identity) and other Gram-negatives (such as *A. tumefaciens*; 42 %), from *S. coelicolor* (62 %), and to putative ADP-Glc pyrophosphorylases

from other high-G+C Gram-positive bacteria, such as *Corynebacterium efficiens* (91 %), *C. diphtheriae* (79 %), *C. jeikeum* (76 %) and *M. tuberculosis* (70 %). An alignment of the deduced amino acid sequence of the *C. glutamicum glgC* gene product with the respective sequences of the *E. coli* enzyme and of some other representative bacteria (Gram-positives and Gram-negatives) is shown in Fig. 3. All the enzymes aligned contain highly conserved stretches, including the *E. coli* ADP-Glc pyrophosphorylase regions known to carry amino acids involved in catalysis or regulation (reviewed by Ballicora *et al.*, 2003). Asp₁₄₂ and Arg₃₂ in the *E. coli* ADP-Glc pyrophosphorylase, proposed to be involved in catalysis, correspond to Asp₁₂₇ and Arg₁₉, respectively, in the *C. glutamicum* enzyme. Lys₁₉₅ and Tyr₁₁₄, known to have specific roles in the binding of substrates (glucose 1-phosphate and ATP, respectively) in the *E. coli* enzyme, correspond to the *C. glutamicum* Lys₁₈₀ and Tyr₁₀₅ residues, and Pro₂₉₅ and Gly₃₃₆, important for the regulation of the *E. coli* enzyme to Pro₂₈₄ and Gly₃₂₃, respectively, in the *C. glutamicum* polypeptide. A sequence motif 'Arg-Ala-Lys-Pro-Ala-Val', which is present in all ADP-Glc pyrophosphorylases (residues 27–32 in the *C.*

<i>C. glutamicum</i>	-----MKGRPNVLAIVLAGGEGKRLFPLTEDRAKPAVFGGTYRLIDFVLSNLVNSGELKIAVLTQYKSHSL	67
<i>M. tuberculosis</i>	-----MREVPVHLGIVLAGGEGKRLYPLTADRAKPAVFGGAYRLIDFVLSNLVNRYLRIQVLTQYKSHSL	67
<i>S. coelicolor</i>	-----MLGIVLAGGEGKRLMPLTADRAKPAVFGGTYRLIDFVLSNLVNGDILRIQVLTQYKSHSL	61
<i>E. coli</i>	MVSLEKNDHMLARQLPLKSVALLAGGRCTRLKDLTNKRAKPAVFGGKFRITIDFALSNCINSIGIRRMCVITQYQSHSL	80
<i>A. tumefaciens</i>	--MSEKR-----VQPLARDAMAVLAGGRGSRKELTDRAKPAVFGGKARTIDFALSNALNSGIRRIQVATQYKAHSL	73
<i>C. glutamicum</i>	DRHISLSWNVSG-PTGQYIASVPAQQRIG-KRWFSGSADAILQSLNLIISDEKPDYVIVFGADHVYRMDPSOMLDEHIASG	145
<i>M. tuberculosis</i>	DRHISQNRVLSG-LAGEYITPVPAAQQRIG-PRWYSGSADAITQSLNLIYDEDPDYIVVFGADHVYRMDPSOMVRFHIDSG	145
<i>S. coelicolor</i>	DRHITTTWRMSS-LLGNVTPVPAQQRIG-PRWELSGSADAILQSLNLVHDEQPEYVAVFGADHVYRMDPSOMLAQHIESG	139
<i>E. coli</i>	VQHIQRGWSFFNEEMNEFVDLLPAQQRMKGENWYRCTADAVTONLDITRRYKAEYVVLVLAGDHIYKQDYSRMLIDHVEKG	160
<i>A. tumefaciens</i>	IRHLQRGWDFFRPERNESFDILPASQVRVSETQWYEGTADAVYQNIIDIEPYAPEYVVLVLAGDHIYKMDYDEYMLQQHVDG	153
<i>C. glutamicum</i>	RAVSAGIRVPREEATAFGCIQSDVDG-NITEFLEKPADPPGTPDDPDMTYASMGNYIFTTEALIQALKDDENNNSDHD	224
<i>M. tuberculosis</i>	AGATVAGIRVPRENATAFGCIDADDG-RIRSFVEKPLEPPGTPDDPDTTFVSMGNYIFTTKVLIDAIRADADDHSDHD	224
<i>S. coelicolor</i>	ACVTVAGIRVPRAESPSFGVITPGSDGQVTGTFLEKPADPPGLADDPGCVFASMGNYVETTKALVEALHRDAEDPDSVHD	219
<i>E. coli</i>	ARCTVACMPVPIEASAFGVMAVDEND-KTIEFVEKPAFPSPMPNDPSKSLASMGIIYVEDADYLYELLEDDRDENSSHD	239
<i>A. tumefaciens</i>	ADVTIGCLEVPRMEATGFGVMHVNEKD-EIIDFLEKPADPPGIPGNEGFALASMGIIYVEHTKFLMEALRRDAADPTSSRD	232
<i>C. glutamicum</i>	MGGDIIPYFVSRNDHVVYDFSGNIVPGAETDRDKGYWRDVGTLDAFYECMDLISVHPPIFNLYNSEWPIIHTTSEGNLPPAK	304
<i>M. tuberculosis</i>	MGGDIIPRLVADGMAAVYDFSDNEVPGAETDRDRAYWRDVGTLDAFYDAHMDLVSHPVFNLYNKRWPPIRGES-NDAPAK	303
<i>S. coelicolor</i>	MGGSIIPQLTDRGEALYDFSANEVPGETTRDQGYWRDVGTLDAYYDAHMDLIAERPAFNLYNRDWPVYTHST-QLSPAR	298
<i>E. coli</i>	FGKDIIPKITEAGLAYAHPFPLSCVQSDPAEP-YWRDVGTLDAYWKANLDLASVVPGLDMYDRNWPIRATYNE-SLPPAK	317
<i>A. tumefaciens</i>	FGKDIIPYIYEHGKAVAHRFADSCVRSDFEHEP-YWRDVGTLDAYWCANIDLTDVVPOLDIYDKSWPIWTYAE-ITPPAK	310
<i>C. glutamicum</i>	FVRG-----GIAOSSVSSGSIISAGTVRNSVLSNNVVVEGATVEGAVLMPCVRIGKGAVVRHAIDLKNNVVVDGELIG	379
<i>M. tuberculosis</i>	FVNG-----GSAQESVVGAGSIISAASVRNSVLSNNVVVDGAIVEGGSVIMPGTRVGRGAVVRHAIDLKNNVVVGPEMVG	378
<i>S. coelicolor</i>	FNAG-----GIASESIIISACCLIRGQVTR-SVLSPGVVVDGAVVQGSVLHDNVHIGRGAVVRCAVLKNNVQVPPGATIG	372
<i>E. coli</i>	FVQDRSGSHGTMNLNSLVSGGCIVISGSVVSQSVLFSRVVRNSFCNIDSAVLLPEVWVGSCRLRRVIDRACVPIEGMVG	397
<i>A. tumefaciens</i>	FVHDDDDRGSASVSSVSGDCIISGAALNRSLLFTGVRANSYSRLNAVVLPSVKIGRHAQLSNVVVIDHGVVPIPEGLIVG	390
<i>C. glutamicum</i>	VDQVRDAQRFKVSAGGVVVVGKNQVV-----	405
<i>M. tuberculosis</i>	VDLEKDRERFAISAGGVVAVGKGVWI-----	404
<i>S. coelicolor</i>	VNPQRDGEYTVSKGGVIALGKQVRP-----	399
<i>E. coli</i>	ENAEEDARRFYRSEEGIVLVTTREMLRKLGHKQER	431
<i>A. tumefaciens</i>	EDPELDAKRRFRTESGICLITQSMIDKLDL----	420

Fig. 3. Alignment of ADP-Glc pyrophosphorylase sequences of *C. glutamicum*, *M. tuberculosis*, *S. coelicolor*, *E. coli* and *A. tumefaciens*. Amino acids identical in three sequences are shaded in black and similar amino acids are shaded in grey.

glutamicum enzyme), has also been shown to be important for activator binding (Greene *et al.*, 1996).

Transcriptional analysis of the *glgC* gene

Northern (RNA) hybridization experiments were performed in order to analyse the size of the *glgC* transcript. Total RNA from *C. glutamicum* WT was hybridized to a 0.37 kb *glgC*-specific digoxigenin-UTP-labelled DNA probe. The hybridization revealed a signal at about 1.3 kb (data not shown), which corresponds well to the size of the *glgC* gene (1.22 kb). A comparison with the genetic organization of *glg* genes in other bacteria reveals that this monocistronic organization is unique among the known bacterial *glgC* genes. In *E. coli*, *A. tumefaciens*, *B. subtilis* and *R. sphaeroides* the *glgC* gene is transcribed together with *glgA* and other *glg* genes in polycistronic operons (Igarashi & Meyer, 2000; Preiss, 1996).

To confirm the presence of a *glgC*-specific promoter and to investigate transcriptional regulation of the *glgC* gene, a transcriptional fusion between the putative *glgC* promoter region and the promoterless chloramphenicol acetyltransferase (CAT) gene was constructed in the promoter probe vector pET2. The resulting plasmid, pET-PC, was transformed into *C. glutamicum* WT and CAT activity was determined in the plasmid-carrying strain during early exponential growth in TY medium and in minimal medium containing glucose, acetate or lactate [each at 1% (w/v)]. Whereas *C. glutamicum* carrying the host plasmid pET2 showed no CAT activity [<0.01 U (mg protein) $^{-1}$] on either medium, *C. glutamicum* (pET-PC) showed activity of 0.10–0.16 U (mg protein) $^{-1}$ when grown in TY medium with or without glucose or in minimal medium containing acetate or lactate. In cells grown in minimal medium containing glucose, CAT activity was about threefold higher [0.40–0.45 U (mg protein) $^{-1}$]. In the course of growth (from 3 to 24 h after inoculation), none of these activities changed significantly. These results confirm the presence of a promoter immediately upstream of the *glgC* gene and indicate weak transcriptional control of the *glgC* gene by the carbon source in the growth medium. In *E. coli*, the expression of the *glgC* gene is subject to positive regulation by cAMP, the cAMP receptor protein CRP and by ppGpp (reviewed by Preiss, 1996). However, in *C. glutamicum* there is no indication of a carbon catabolite repression mechanism involving cAMP/CRP or involving a catabolite control protein (CcpA) and a catabolite-responsive element (CRE), the typical regulatory elements in carbon catabolite repression of low-G+C Gram-positive bacteria (Hueck *et al.*, 1994; Stülke & Hillen, 2000). Also, there is no indication for an involvement of the stringent response since a *C. glutamicum* *rel* gene deletion mutant, unable to synthesize (p)ppGpp, showed unaltered *glgC* expression when compared to the wild-type strain (Brockmann-Gretza & Kalinowski, 2006).

The threefold higher promoter activity in the cells grown in glucose medium instead of acetate or lactate medium did

not correspond to the 20-fold higher ADP-Glc pyrophosphorylase activities observed in glucose-grown cells when compared to cells grown on the other carbon sources (see above). This result suggests that the observed carbon-source-dependent regulation of ADP-Glc pyrophosphorylase activity is only to a small extent due to transcriptional regulation of the *glgC* gene and mainly due to post-transcriptional control at the RNA or at the enzyme level. In *E. coli* *glgC* gene expression has been shown to be post-transcriptionally regulated by the CsrA protein, by acceleration of *glg* transcript degradation (Liu *et al.*, 1995). Taken together, the data suggest that *glgC* in *C. glutamicum* is under transcriptional and post-transcriptional control; however, the nature and the mechanisms of *glgC* expression control need to be investigated in future studies.

Inactivation of the chromosomal *glgC* gene and characterization of the mutant

To study whether *C. glutamicum* requires the *glgC* gene for growth, for ADP-Glc pyrophosphorylase activity and for intracellular glycogen accumulation, the chromosomal *glgC* gene was inactivated by integration mutagenesis (see Methods). The resulting strain, *C. glutamicum* WT-IMC, was then analysed in comparison to the WT *C. glutamicum*. Both *C. glutamicum* WT and *C. glutamicum* WT-IMC grew equally well (measured by doubling time and final optical density) in LB and TY complex medium and in minimal medium containing glucose, fructose, sucrose, acetate or lactate (not shown). Thus, an intact *glgC* gene is not required for growth in any of these media. In contrast, inactivation of *glgC* led to a loss of the vast majority of ADP-Glc pyrophosphorylase activity [0.4 mU (mg protein) $^{-1}$ in the mutant instead of 6 mU (mg protein) $^{-1}$ in the WT strain] and to a complete loss of intracellular glycogen in all media tested (data not shown). These results indicate (i) that *glgC* in fact encodes an ADP-Glc phosphorylase and (ii) that a functional *glgC* gene is required for intracellular glycogen accumulation in *C. glutamicum*. The low residual activity in the ADP-Glc pyrophosphorylase assay with *C. glutamicum* WT-IMC cells might be due to an ADP-Glc pyrophosphatase (EC 3.6.1.21) activity, which releases sugar 1-phosphate from ADP-sugar. The *cg1607* gene, encoding a protein with similarity (25% identity) to the *E. coli* ADP-Glc pyrophosphatase (Moreno-Bruna *et al.*, 2001) and carrying the typical 'nudix' motif (Mildvan *et al.*, 2005), has been annotated as a pyrophosphohydrolase in the *C. glutamicum* genome (Kalinowski *et al.*, 2003).

We also studied growth, substrate consumption and glycogen content of *C. glutamicum* WT and the *glgC* mutant WT-IMC in glucose-minimal medium in a controlled parallel fermentation system providing a constant pH of 7.0 and a constant oxygen concentration of 30% saturation. *C. glutamicum* WT and WT-IMC grew at nearly identical growth rates of 0.36 h $^{-1}$ and reached approximately the same final OD₆₀₀ of 35 (Fig. 4a). At the beginning of the exponential growth phase, glucose was consumed considerably faster by *C. glutamicum* WT than by the *glgC*

Fig. 4. Growth and glucose consumption (a) and intracellular glycogen content (b) of *C. glutamicum* WT (filled symbols) and *C. glutamicum* WT-IMC (open symbols) in minimal medium containing 2% glucose. Growth is indicated by circles, glucose consumption by squares, and glycogen content by triangles. The cellular glycogen content at 4, 8, 12 and 24 h was also analysed by TLC (c). Dot M represents the glycogen standard.

mutant (Fig. 4a). This faster glucose consumption of the WT strain was paralleled by extensive glycogen formation (Fig. 4b). The glycogen content of the WT was maximal in the early exponential growth phase and then sharply decreased. Twelve hours after inoculation, the glucose in both cultures was exhausted (Fig. 4a), and quantitative enzymic analysis of the glycogen content as well as qualitative analysis of intracellular carbohydrates via TLC (Fig. 4c) showed almost complete degradation of the intracellular glycogen in *C. glutamicum* WT. In contrast, the mutant strain *C. glutamicum* WT-IMC formed no glycogen at any time tested (Fig. 4b, c).

To test for an effect of *glgC* inactivation and of the inability to form intracellular glycogen on the viability of *C. glutamicum* in the stationary phase, we performed plate count experiments. When compared to *C. glutamicum* WT, culture aliquots of WT-IMC, taken 24 h, 48 h and 148 h after inoculation and incubation in minimal medium containing 2% (w/v) glucose at 30 °C, contained the same number of viable cells (about 5×10^8 , 2×10^8 and 1×10^8 cells ml⁻¹, respectively). Thus, the inability to form glycogen had no effect on the viability of *C. glutamicum* in the stationary phase.

Direct competition experiments in minimal glucose medium with *C. glutamicum* WT and the WT-IMC mutant were performed to test for a possible growth advantage of the WT strain. Both strains were inoculated together at different ratios (100%, 80%, 50% and 20% *C. glutamicum* WT-IMC), consecutively grown in minimal

medium with 2% (w/v) glucose, and the percentage of the mutant was determined in the course of the cultures. No changes were observed in the ratio between the WT and the mutant strain WT-IMC in the course of three consecutive cultures. This result shows that the WT strain is not able to outcompete the *glgC* mutant in minimal glucose medium and thus suggests that glycogen formation is not advantageous under the conditions tested.

In summary, the results of the characterization of the mutant indicate (i) that the *glgC* gene in *C. glutamicum* in fact encodes a protein with ADP-Glc pyrophosphorylase activity, (ii) that ADP-Glc pyrophosphorylase of *C. glutamicum* is involved in intracellular glycogen formation, (iii) that intracellular glycogen formation is not essential for growth of *C. glutamicum* in any medium tested and (iv) that the inability to form intracellular glycogen is no disadvantage for *C. glutamicum* under all conditions tested here.

Significance of glycogen synthesis for amino acid production

As *C. glutamicum* is used for the industrial production of L-glutamate and L-lysine from glucose, it was interesting to investigate the influence of the inactivation of glycogen synthesis on amino acid formation. In standard glutamate fermentations in minimal medium containing 2% glucose and using ethambutol as the trigger *C. glutamicum* WT and WT-IMC accumulated about the same concentration of glutamate (22.1 and 19.0 mM, respectively). To analyse the significance of glycogen formation for L-lysine production,

we constructed a *glgC*-negative derivative of the lysine producer *C. glutamicum* DM1730. The resulting mutant *C. glutamicum* DM1730-IMC accumulated approximately the same concentration of L-lysine as the parental strain DM1730 (36.7 and 38.9 mM, respectively). These results indicate that the absence of glycogen synthesis does not significantly affect L-glutamate and L-lysine production by *C. glutamicum*.

Role of glycogen synthesis in osmoprotection of *C. glutamicum*

For *C. glutamicum*, adaptation to hyperosmotic stress caused by changes in the osmolality of the environment is necessary for survival in its natural habitat, soil. The organism counteracts shifts to high osmolality by the synthesis of compatible solutes, such as glutamate, proline and trehalose (Wolf *et al.*, 2003). The TreYZ pathway, one of two major pathways leading to trehalose formation in *C. glutamicum* (Wolf *et al.*, 2003; Tzvetkov *et al.*, 2003), starts from linear maltodextrins, which are intermediates in glycogen synthesis. To investigate the influence of glycogen synthesis on growth during hyperosmotic stress, *C. glutamicum* WT and mutant WT-IMC were cultivated in minimal medium with glucose and 750 mM NaCl. Compared to the WT strain, the mutant showed a lag phase of about 2 h and then commenced to grow with a rate of about 0.22 h^{-1} . Both strains grew to approximately the same final OD₆₀₀, 30.5 and 29.8, respectively (Fig. 5a). In the early stages of growth, glucose consumption by the WT strain was slightly higher than by the mutant; however, glucose was used up by both strains within 14 h (Fig. 5a). No glycogen was formed throughout growth by the mutant strain WT-IMC (Fig. 5b). The glycogen formation profile of the WT, however, showed differences when compared to that during growth of this strain in minimal medium without NaCl (Fig. 4b and 5b, respectively). The maximal glycogen level of about 50 mg glucose (g dry weight)⁻¹ was

observed in the middle of the exponential growth phase and the degradation of glycogen was significantly slower (Fig. 5b).

Our results show that *C. glutamicum* cells devoid of glycogen synthesis in principle are able to cope with hyperosmotic conditions; however, for fast adaptation to such conditions, and thus for better competition in nature, intracellular glycogen formation obviously gives advantages. This result can be explained by the previous finding that the *treY* and *treZ* genes, encoding maltotriose synthase (TreY) and maltotriose trehalohydrolase (TreZ), are constitutively expressed whereas the expression of the *otsA* gene, encoding the trehalose-6-phosphate synthase of the alternative OtsAB trehalose synthetic pathway, was low in basal medium and upregulated in response to a hyperosmotic shock by the addition of 750 mM NaCl (Wolf *et al.*, 2003). Thus, the availability of maltodextrins (due to the synthesis of glycogen) and the constitutive presence of TreY and TreZ guarantee an immediate response to osmotic stress, whereas in the absence of either glycogen synthesis or the TreYZ pathway, the enzymes for the alternative trehalose synthetic pathways (OtsA and OtsB) have to be formed, and thus the trehalose response of *C. glutamicum* to hyperosmotic conditions takes some time. That the OtsAB pathway principally can substitute the TreYZ pathway for trehalose synthesis became clear from studies with single and/or double mutants defective in either or both of the pathways (Wolf *et al.*, 2003; Tzvetkov *et al.*, 2003) and from *treYZ* and *otsAB* overexpression studies, which aimed at improvement of microbiological trehalose production with *C. glutamicum* (Padilla *et al.*, 2004a; Carpinelli *et al.*, 2006).

Taken together, the results indicate that glycogen accumulation in *C. glutamicum* is favourable for fast adaptation to hyperosmotic stress under the conditions tested. Since hyperosmotic stress is also a factor in large-scale amino acid fermentations, glycogen accumulation might also be of

Fig. 5. Growth and glucose consumption (a) and intracellular glycogen content (b) of *C. glutamicum* WT (filled symbols) and *C. glutamicum* WT-IMC (open symbols) in minimal medium containing 2% glucose and 750 mM NaCl. Growth is indicated by circles, glucose consumption by squares and glycogen content by triangles.

advantage for the industrial amino acid production process. It should be noted here, however, that the significance of trehalose as a compatible solute in *C. glutamicum* depends on environmental conditions (Guillouet & Engasser, 1995; Wolf *et al.*, 2003; Tzvetkov *et al.*, 2003) and also that other compatible solutes (such as proline) are involved in the response of *C. glutamicum* to hyperosmotic conditions. Thus, the relevance of glycogen synthesis for fast osmoprotection might be restricted to conditions in which trehalose represents the main compatible solute.

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