Roles of maltodextrin and glycogen phosphorylases in maltose utilization and glycogen metabolism in Corynebacterium glutamicum

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Corynebacterium glutamicum transiently accumulates large amounts of glycogen, when cultivated on glucose and other sugars as a source of carbon and energy. Apart from the debranching enzyme GlgX, which is required for the formation of maltodextrins from glycogen, α-glucan phosphorylases were assumed to be involved in glycogen degradation, forming α-glucose 1-phosphate from glycogen and from maltodextrins. We show here that C. glutamicum in fact possesses two α-glucan phosphorylases, which act as a glycogen phosphorylase (GlgP) and as a maltodextrin phosphorylase (MalP). By chromosomal inactivation and subsequent analysis of the mutant, cg1479 was identified as the malP gene. The deletion mutant C. glutamicum ΔmalP completely lacked MalP activity and showed reduced intracellular glycogen degradation, confirming the proposed pathway for glycogen degradation in C. glutamicum via GlgP, GlgX and MalP. Surprisingly, the ΔmalP mutant showed impaired growth, reduced viability and altered cell morphology on maltose and accumulated much higher concentrations of glycogen and maltodextrins than the wild-type during growth on this substrate, suggesting an additional role of MalP in maltose metabolism of C. glutamicum. Further assessment of enzyme activities revealed the presence of 4-α-glucanotransferase (MalQ), glucokinase (Glk) and α-phosphoglucomutase (α-Pgm), and the absence of maltose hydrolase, maltose phosphorylase and β-Pgm, all three known to be involved in maltose utilization by Gram-positive bacteria. Based on these findings, we conclude that C. glutamicum metabolizes maltose via a pathway involving maltodextrin and glucose formation by MalQ, glucose phosphorylation by Glk and maltodextrin degradation via the reactions of MalP and α-Pgm, a pathway hitherto known to be present in Gram-negative rather than in Gram-positive bacteria.

INTRODUCTION

Corynebacterium glutamicum is a Gram-positive soil bacterium employed in the production of various amino acids (Eggeling & Bott, 2005), and serves as a model organism for the mycolic acid-containing suborder Corynebacteriaceae, which includes pathogenic species such as Corynebacterium diphtheriae, Mycobacterium tuberculosis and Mycobacterium leprae. The organism grows on a variety of mono- and disaccharides, alcohols and organic acids as single or combined sources of carbon and energy (Liebl, 2005), and transiently forms intracellular glycogen when cultivated on sugar substrates (Tzvetkov et al., 2003; Seibold et al., 2007). We recently showed that C. glutamicum forms maltodextrins in the course of glycogen degradation (Seibold & Eikmanns, 2007), and thus we speculate that glycogen is degraded in C. glutamicum by a pathway which proceeds similarly or identically to the one present in Escherichia coli. This pathway involves the interplay of six enzymes (Fig. 1). Glycogen phosphorylase (GlgP) cleaves α-1,4-glycosidic bonds at the non-reducing ends of glycogen and forms glucose 1-phosphate and phosphorylase-limited dextrins (pl-dextrins) (Dauvillee et al., 2005; Alonso-Casajus et al., 2006). Cleavage of the α-1,6-glycosidic bonds by the debranching enzyme GlgX leads to the formation of maltodextrins, which are then further degraded by maltodextrin phosphorylase (MalP) and maltodextrin glucosidase (MalZ) (Dippel et al., 2005). MalP and MalZ are also involved in maltose metabolism in E. coli, since in the course of maltose utilization, various maltodextrins are formed by 4-α-glucanotransferase (MalQ; see Fig. 1). This enzyme catalyses the transfer of maltosyl and longer dextrinyl residues from intracellular maltodextrins such as maltotriose onto the maltose, which is taken up by a high-affinity binding-protein-dependent
ABC transporter (Dippel et al., 2005; Daus et al., 2006; Chen et al., 2003). Thereby, MalQ forms larger maltodextrins and releases glucose (see Fig. 1) (Monod & Torriani, 1950; Pugsley & Dubreuil, 1988). Like the maltodextrins formed in the course of glycogen degradation, the MalQ-generated maltodextrins are degraded by either MalP or MalZ. Glucose 1-phosphate derived from the GlgP and MalP reactions is converted into glucose 6-phosphate by α-phosphoglucomutase (α-Pgm; Lu & Kleckner, 1994; Adhya & Schwartz, 1971), the glucose derived from the MalQ and MalZ reactions is phosphorylated by glucokinase (Glk) to glucose 6-phosphate (Meyer et al., 1997).

The maltose metabolism of low-GC Gram-positive bacteria, e.g. lactic acid bacteria such as *Streptococcus bovis*, *Lactococcus lactis*, *Lactobacillus sanfrancensis* and *Enterococcus faecalis* (Ehrmann & Vogel, 1998; Nilsson & Radström, 2001; Le Breton et al., 2005) or the endospore-forming *Bacillus subtilis* (Schönert et al., 2006), has been thoroughly investigated, and proceeds by different pathways, which, unlike the pathway present in *E. coli* (Fig. 1), generally do not include reactions that form maltodextrins. In *S. bovis*, maltose is taken up by a permease and directly cleaved into two glucose molecules by maltose hydrolase (Martin & Russell, 1987; Andersson & Radström 2002). In *L. lactis* and *Lb. sanfrancensis*, maltose is also taken up by a permease; however, the intracellular maltose is then cleaved by a maltose phosphorylase into glucose plus β-glucose 1-phosphate (Nilsson & Radström, 2001; Ehrmann & Vogel, 1998). In Ent. *faecalis*, maltose uptake is accomplished by a phosphotransferase system (PTS); the maltose 6-phosphate thereby formed is cleaved by maltose-6-phosphate phosphorylase to glucose 6-phosphate and β-glucose 1-phosphate (Le Breton et al., 2005). The conversion of β-glucose 1-phosphate to glucose 6-phosphate is catalysed in these lactic acid bacteria by β-phosphoglucomutase (β-Pgm) (Levander et al., 2001; Ehrmann & Vogel, 1998; Le Breton et al., 2005). In *B. subtilis*, the uptake of maltose and of maltodextrins is accomplished by a maltose-specific PTS and a maltodextrin-specific ATP-binding cassette (ABC) transporter, respectively (Schönert et al., 2006). The maltose 6-phosphate derived from the uptake of maltose by the PTS is hydrolysed by a maltose-6-phosphate hydrolase, GlvA, resulting in the formation of glucose 6-phosphate and glucose (Thompson et al., 1998). Maltodextrins are degraded by the concerted action of cytoplasmic maltogenic amylase YvdF, maltose phosphorylase YvdK and α-glucosidase MalL, forming glucose and β-glucose 1-phosphate (Cho et al., 2000; Schönert et al., 2006). Glucose 6-phosphate is then generated from glucose by glucose kinase Glk (Skratlas & Dahl, 1998) or from β-glucose 1-phosphate by PgcM, a β-phosphoglucomutase, and glucose-1-phosphate diphosphatase (Mesak & Dahl, 2001). In contrast to the situation in all the above-mentioned Gram-positive bacteria, maltodextrin and maltose metabolism has scarcely been investigated in *C. glutamicum* and other members of the suborder *Corynebacterianeae*. This is surprising, since maltose serves as an excellent substrate for the cultivation of *C. glutamicum*. Moreover, intracellular maltose and maltodextrins are the substrates of the trehalose synthesis pathways via TreYZ and TreS, both of which have been thoroughly investigated in *C. glutamicum* and mycobacteria (De Smet et al., 2000; Wolf et al., 2003;...
To investigate glycogen, maltodextrin and maltose metabolism, we here firstly analysed *C. glutamicum* for α-glucan phosphorylase activities. We show that *C. glutamicum* indeed possesses both a MalP enzyme, encoded by cg1479, and a GlpP enzyme, encoded by cg2289. Unlike in other Gram-positive bacteria, MalP in *C. glutamicum* is involved not only in glycogen degradation but also in maltose metabolism. Furthermore, we show that the enzymes known to be involved in maltose metabolism in *E. coli* are also present in *C. glutamicum*, indicating that glycogen, maltose and maltodextrin metabolism in this organism is different from that known to be present in other Gram-positive bacteria.

**METHODS**

**Bacterial strains, plasmids and culture conditions.** The bacteria used in this study were *E. coli* DH5α (Hanahan, 1983) and *C. glutamicum* wild-type (WT) (ATCC 13032, American type Culture Collection). Plasmid pK19mobsecB (Scharf et al., 1994) was employed for construction of *C. glutamicum* ΔmalP, and plasmid pEKe2 (Eikmanns et al., 1991a) for construction of pEKe2-glcp2. *E. coli* and all pre-cultures of *C. glutamicum* were grown aerobically in TY complex medium (Sambrook, 2001) at 37 and 30 °C, respectively, as 60 ml cultures in 500 ml baffled Erlemeyer flasks on a rotary shaker at 120 r.p.m. For the main cultures of *C. glutamicum*, cells of an overnight pre-culture were washed with 0.9% (w/v) NaCl (saline) and then inoculated into minimal medium (Eikmanns et al., 1991b) containing glucose or maltose at the concentrations indicated in Results. Cultivation was done aerobically at 30 °C as 250 ml cultures in a ‘feedbatch-pro’ parallel fermentation system from DASGIP, as described previously (Seibold et al., 2007). 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 measurement of OD600, additional live-cell counts were performed using LB agar plates (Sambrook et al., 2001). The dry weight was determined by vacuum filtration using pre-weighed, dried glass fibre membranes (Millipore); 1–2 ml culture was filtered, the filters were washed twice with 2.5 ml saline, dried for 24 h at 60 °C, and weighed again. Online analysis of the CO2 content of the exhaust gas was performed using a GA4 gas analyser from DASGIP, as described previously (Blombach et al., 2007).

**Analysis of intracellular carbohydrates.** For enzymic analysis of intracellular polysaccharides, 5 ml samples of the respective cultures were harvested, cell extracts were prepared and glycogen was determined with amyloglucosidase as described previously (Seibold et al., 2007). TLC was used to distinguish between glycogen and other carbohydrates. Sample preparation, application, separation and visualization of the carbohydrates on the TLC plate was performed as described previously (Seibold et al., 2006). Carbohydrates were identified by comparison to the migration of authentic standards, i.e. glucose, maltose, maltotriose, maltoheptaose and glycogen (all except glucose obtained from Sigma Aldrich; glucose was obtained from Roth).

**Quantification of glucose and maltose.** For analysis of the glucose or maltose concentrations 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 of sugars with a Hitachi HPLC system equipped with a refractive index detector (L2490) and a Nucleo Sugar 810H column (Macherey–Nagel). The mobile phase was 0.01 M H2SO4 with a flow rate of 0.5 ml min⁻¹, and the column temperature was 40 °C.

**Enzyme assays.** All enzyme activities were assayed in a final volume of 1 ml by spectrophotometric measurement of the variation in the NADP(H) concentration at 340 nm at 30 °C. All specific enzyme activities given in Results are means of at least two independent cultivations and three technical replicates; SDs in all assays were below 10%. Cell extracts for all enzymic measurements were prepared as follows: *C. glutamicum* cells were harvested at selected time points, washed twice in 0.1 M Tris/HCl (pH 7.4), 20 mM KCl, 5 mM MgSO4 and resuspended in 0.75 ml of the same buffer containing 0.1 mM EDTA, 2 mM DTT and 10% (v/v) glycerol. The cell suspension was transferred to 2 ml screw-cap vials together with 250 mg glass beads (150–212 μm, Sigma) and subjected five times for 30 s to mechanical disruption with a RiboLysyer (Hybird) 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), possibly serving as a substrate for background glucose and glucose 1-phosphate formation, were removed using an Äkta Purifier System (Amersham Bioscience) by separation with a HiTrapDesalting column (Amersham Bioscience) equilibrated with 0.1 M Tris/HCl (pH 7.4), 25 mM MgCl2. Protein concentrations were determined using the bichoninic acid protein assay reagent kit (Pierce) with BSA as the standard.

4-z-Glucanotransferase activity (MalQ) was assayed by the production of maltodextrins and glucose from maltotriose and maltose, essentially as described by Xavier et al. (1999). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 25 mM MgCl2, 2 mM NADP, 2 mM ATP, 10 mM maltose, 10 mM maltotriose, 2 U hexokinase and 2 U glucose-6-phosphate dehydrogenase (Roche Diagnostics). Activity was measured spectrophotometrically by following the formation of NADPH at 340 nm after addition of maltotriose. For the identification of all products formed by the MalQ reaction, a discontinuous assay, containing 50 mM potassium phosphate buffer (pH 7.0), 25 mM MgCl2, 10 mM maltose and 10 mM maltotriose was used. After 30 min of incubation at 30 °C, the reaction was stopped by heat inactivation (10 min, 95 °C). Afterwards, the products formed were analysed by TLC as described above.

Glucokinase (Glk) activity in cell-free extracts was determined according to the method of Park et al. (2000) by measuring the formation of NADPH in a coupled enzyme assay containing 50 mM potassium phosphate buffer (pH 7.0), 20 mM glucose, 2 mM ATP, 25 mM MgCl2, 2 mM NADP and 2 U glucose-6-phosphate dehydrogenase.

β-Phosphoglucomutase (β-Pgm) activity was analysed as described by Andersson & Radstrom (2002). The conversion from β-glucose 1-phosphate to glucose 6-phosphate was assayed in a coupled reaction with glucose-6-phosphate dehydrogenase at 30 °C. The reaction mixture was composed of 100 mM Tris/HCl (pH 7.4), containing 10 mM MgCl2, 1 mM NADP, 2 U glucose-6-phosphate dehydrogenase and 2 mM β-glucose 1-phosphate as the substrate. The formation of NADPH was measured spectrophotometrically at 340 nm.

α-Phosphoglucomutase (α-Pgm) was assayed according to the method of Dominguez et al. (1998), essentially as described for β-Pgm (see above), using 1 mM α-glucose 1-phosphate instead of β-glucose 1-phosphate as a substrate.
Maltose hydrolyase and maltose phosphorylase catalyse the formation of glucose from maltose. Glucose formation from maltose was measured in a coupled assay with hexokinase and glucose-6-phosphate dehydrogenase by following NADPH formation at 340 nm. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 10 mM MgCl₂, 2 mM NADP, 2 mM ATP, 2 U hexokinase and 2 U glucose-6-phosphate dehydrogenase. After 2 min preincubation without substrate, the reaction was started by addition of 5 mM maltose and the reduction of NADP was monitored spectrophotometrically at 340 nm.

The activity of maltodextrin phosphorylase (MalP) was measured in a continuous assay, essentially as described by Xavier et al. (1999), at 30 °C with maltotetraose as the substrate. The assay mixture contained 50 mM potassium phosphate buffer (pH 7.0), 10 mM MgCl₂, 2 mM NADP, 2 U phosphoglucomutase (Roche) and 2 U glucose-6-phosphate dehydrogenase. After 2 min preincubation, the assay was started by the addition of 5 mM maltotetraose and the reduction of NADP was monitored. For the determination of the substrate specificity, experiments were performed with maltose, maltopentaose or maltotetraose as substrate.

Maltodextrin glucosidase (MalZ) activity was measured in a continuous assay at 30 °C, similar to the assay described for MalP. The same reaction mixture was used, except that maltotetraose was used as the standard substrate and 2 mM ATP and 2 U hexokinase were added instead of phosphoglucomutase.

Glycogen phosphorylase (GlgP) activity was assayed as described for MalP (see above) with glycogen instead of maltotetraose as the substrate.

**DNA isolation, transfer and manipulations.** Standard procedures were employed for plasmid isolation, cloning and transformation of E. coli DH5α, as well as for electrophoresis (Sambrook et al., 2001). C. glutamicum chromosomal DNA was isolated according to the protocol of Eikmanns et al. (1994). Transformation of C. glutamicum was performed by electroporation using the methods of Tauch et al. (2002); the recombinant strains were selected on LB-BHIS agar plates containing kanamycin (25 μg ml⁻¹). Electroploration of E. coli was performed according to the method of Dower et al. (1988). 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 cycler (Biotron). Deoxyxynucleoside triphosphates were obtained from MBI-Fermentas, and oligonucleotides (primers) from biomers.net. Cycling times and temperatures were chosen according to fragment length and primer constitution. PCR products were separated on agarose gels and purified using the Nucleospin Extract II kit (Macherey-Nagel).

**Construction of the C. glutamicum malP deletion mutant.** Inactivation of the chromosomal malP gene in C. glutamicum was performed using crossover PCR and the suicide vector pK19mobac8. malP-specific DNA fragments were generated using the primer pairs MalPDelFor1 (5’-CGGAATTCGAGGATGATCTCAGAGCATTATGAT-3’, EcoRI site underlined) and MalPDelRev1 (5’-ACGAATTCGGATATGATCCATAGCGGATTGCATGTTAG-3’, BamHI site underlined). The recombinant strains were selected on LB agar plates containing kanamycin (25 μg ml⁻¹). The hybridization resulted in one signal of about 3.6 kb for C. glutamicum AmalP. These sizes were expected for the WT strain and the malP deletion mutant, respectively.

**Microscopic imaging.** For phase-contrast and fluorescence microscopy, 2 μl of a culture sample was placed on a microscope slide coated with a thin agarose (1 %) layer and covered by a coverslip. Viability staining was performed using the Live/Dead BacLight Bacterial Viability kit (Molecular Probes) according to the manufacturer’s instructions. To control the staining properties of the kit for C. glutamicum, controls were performed as instructed by the supplier. In brief, 1 ml samples of exponentially growing C. glutamicum WT cells were harvested by centrifugation, washed once with saline solution, and incubated for 30 min with either saline (for live bacteria) or 70 % isopropyl alcohol (for dead bacteria). Afterwards, the cells were pelleted, suspended in 1 ml saline, stained and observed. Images were taken on a Zeiss AxioImager M1, equipped with a Zeiss AxioCam HRm camera. Generally, an EC Plan-Neofluar 100 x/1.3 Oil Ph3 objective was used. Digital images were acquired with the AxioVision (Zeiss) software. Final image preparation was done in Adobe Photoshop 6.0.

**RESULTS**

**Activities of GlgP and MalP in C. glutamicum**

To complete our previous investigations of glycogen degradation in C. glutamicum (Seibold & Eikmanns, 1994), the complete chromosomal malP gene was deleted via homologous recombination (double crossover). Screening of the malP mutants was performed on LB agar plates containing 10 % (w/v) sucrose. The deletion at the chromosomal locus of potential candidates was verified by PCR using primers MalPDelFor1 and MalPDelRev2 and by Southern blot analysis. As a probe, a 679 bp fragment upstream of malP was amplified from chromosomal DNA of C. glutamicum WT using primers MalPDelFor1 and MalPDelRev1. Labelling with DIG-DUTP, hybridization, washing and detection were conducted using the nonradioactive DIG DNA Labeling and Detection Kit (Roche) according to the manufacturer’s instructions. The labelled probe was hybridized to BanHI-restricted and size-fractionated chromosomal DNA from C. glutamicum WT and the potential deletion mutant C. glutamicum AmalP. The hybridization resulted in one signal of about 2.9 kb for C. glutamicum WT and one signal of about 3.6 kb for C. glutamicum AmalP. These sizes were expected for the WT strain and the malP deletion mutant, respectively.

**Overexpression of glgP in C. glutamicum.** To achieve overexpression of glgP, plasmid pEKE2-glGp was constructed. For this purpose, glgP (cg2289) was amplified by PCR using the primers OEglgP-for (5’-ACGCGTTCACATCAGAGCTTAC-3’, SalI site underlined) and OEglgP-rev (5’-CGGATCCAAATTCGGCTTGCATCTAGAC-3’, EcoRI site underlined). Using the PCR-generated 5’ SalI and 3’ EcoRI restriction sites of the PCR product, the construct was ligated into pEKE2 and transformed into E. coli. The recombinant plasmid was isolated from E. coli and electroporated into C. glutamicum WT, resulting in C. glutamicum (pEKE2-glGp). For induction of the glgP gene, 0.5 mM IPTG was added to the culture.

**Computational analysis.** Database searches and alignments were carried out by using BLAST (Altschul et al., 1990) and CLUSTAL W (Thompson et al., 1994). The GenBank accession numbers for the annotated genome sequences for C. glutamicum are NC_006958 and NC_003450 (Kalinowski et al., 2003; Ikeda & Nakagawa, 2003). The following NCBI-GL accession numbers for protein sequences were retrieved from the KEGG database (Kanehisa et al., 2006): 49176351, E. coli maltodextrin phosphorylase; 16131302, E. coli glycogen phosphorylase; 16131292, E. coli 4-α-glucanotransferase; 16128664, E. coli α-phosphoglucomutase; 9798607, Thermus aquaticus α-glucan phosphorylase; 2660639, Thermotoga maritima α-glucan phosphorylase.
2007) we analysed extracts of C. glutamicum cells cultivated in minimal medium with glucose as sole carbon source for GlgP activity. Independent of the growth phase, we observed specific GlgP activities of about 0.05 U (mg protein)$^{-1}$. The specific GlgP activities were about 50% lower in cells cultivated with maltose as the sole carbon source [0.02 U (mg protein)$^{-1}$ in mid-exponential and in early stationary growth phases].

We next analysed C. glutamicum cell extracts for MalP activity and found an activity that was slightly higher in extracts from cells growing exponentially on maltose [0.20 U (mg protein)$^{-1}$] than in extracts from cells growing exponentially on glucose [0.18 U (mg protein)$^{-1}$]. The specific activities were highest in samples from early stationary growth phase; however, in this phase no differences were observed in activity between maltose- and glucose-grown cells [0.25 U (mg protein)$^{-1}$]. Substrate specificity of MalP was assayed by use of various maltodextrins. The activities did not vary significantly when maltohexaose or maltopentaose was used as a substrate; however, with maltotetraose the specific activity was lower, i.e. 0.09 U (mg protein)$^{-1}$ in samples from the mid-exponential and 0.15 U (mg protein)$^{-1}$ in samples from the early stationary growth phase. Thus, MalP of C. glutamicum obviously possesses a preference for maltodextrins with at least five glucose residues.

**MalP of C. glutamicum is encoded by cg1479**

In the genome sequence of C. glutamicum, there are two genes that encode proteins with similarity to MalP and/or GlgP enzymes from other organisms, i.e. cg1479, annotated as glgP1, and cg2289, annotated as glgP2 (Kalinowski et al., 2003). Comparison of the amino acid sequence from E. coli MalP with those of the deduced proteins from the C. glutamicum genome revealed an identity of 41% with Cg1479. However, this protein also shares 40% identity with GlgP from E. coli. The deduced amino acid sequence of cg2289 shows 40 and 36% identity with z-glucan phosphorylases from Thermus aquaticus and Thermotoga maritima, respectively (Takahara et al., 2001; Bibel et al., 1998).

To identify the physiological function and activity of the Cg1479 protein, we inactivated the corresponding gene by chromosomal deletion and analysed the resulting C. glutamicum mutant for MalP and GlgP activities. In fact, cell extracts of the cg1479 mutant completely lacked MalP activity, whereas WT cells showed an activity of 0.25 U (mg protein)$^{-1}$ (see also above). In contrast, the specific GlgP activity in cg1479 mutant cells was about the same as that in cells of C. glutamicum WT [0.04 U (mg protein)$^{-1}$]. From these results it can be concluded that cg1479 in fact encodes the MalP protein of C. glutamicum and therefore we refer to it in the following sections as malP. Accordingly, the cg1479 mutant was designated C. glutamicum ΔmalP.

We also tried to inactivate cg2289 by either deletion or integration; however, for unknown reasons, we did not succeed in obtaining such mutants. Therefore, we constructed C. glutamicum (pEKEx2-eglP), which expresses cg2289 under control of the tac promoter. Indeed, the specific GlgP activity in cell extracts of C. glutamicum (pEKEx2-eglP) was about 50% higher than that of the WT strain [0.06 and 0.04 U (mg protein)$^{-1}$, respectively], whereas the specific MalP activity was not altered. From these results we conclude that cg2289 probably encodes the GlgP of C. glutamicum and therefore we refer to it in the following text as glgP.

**Growth and glycogen content of C. glutamicum ΔmalP**

To test for physiological consequences of malP inactivation in C. glutamicum, we analysed the growth and glycogen content of C. glutamicum ΔmalP in minimal medium with either glucose or maltose as carbon source. When cultivated with 2% glucose, no differences in growth between C. glutamicum WT and C. glutamicum ΔmalP were observed (Fig. 2a). However, whereas the glycogen content of C. glutamicum WT dropped during the exponential growth phase, a significantly slower glycogen decrease was observed in C. glutamicum ΔmalP (Fig. 2b). When cultivated with 2% maltose, the growth behaviour of C. glutamicum ΔmalP was significantly altered in comparison with that of C. glutamicum WT (Fig. 2c). In the mid-exponential phase, the growth rate of the mutant strain dropped drastically from 0.33 h$^{-1}$ initially to 0.05 h$^{-1}$ and the final OD$_{600}$ was slightly lower in comparison with the WT strain. The slower growth of C. glutamicum ΔmalP was in accordance with the CO$_2$ concentration in the exhaust air quantified. During the first 5 h of growth the specific rate of maltose utilization was basically the same for C. glutamicum WT and C. glutamicum ΔmalP, and remained roughly constant for the WT until the substrate was consumed (Fig. 2c). In contrast, maltose utilization of the ΔmalP mutant decreased in the mid-exponential growth phase. However, the maltose was completely consumed (Fig. 2c). Major differences were also observed in the polysaccharide content of C. glutamicum ΔmalP compared with C. glutamicum WT. The mutant accumulated nearly twice as much intracellular carbohydrate as the WT (see Fig. 2d). Whereas the polysaccharide content decreased in C. glutamicum WT after reaching its maximum at the mid-exponential growth phase, it increased continuously throughout the growth of the malP mutant and only when entering the stationary growth phase did it slowly decrease.

TLC analysis of the extracts from maltose-grown cells revealed that in C. glutamicum WT various maltodextrins were present in samples from the mid-exponential growth phase which were not detected during the further progress of cultivation (Fig. 3). No intracellular carbohydrates were detected in samples from C. glutamicum WT taken 12, 24
and 48 h after inoculation. Samples from *C. glutamicum ΔmalP* also contained various polysaccharides apart from maltose and glycogen. However, in contrast to the samples from *C. glutamicum* WT, even 24 h after inoculation maltodextrins and glycogen were detected in the mutant cells. The spots corresponding to maltodextrins vanished at later time points.

**Morphology and viability of *C. glutamicum ΔmalP***

Microscopic analysis revealed no significant differences in cell shape for *C. glutamicum ΔmalP* during growth on glucose and for the WT strain throughout cultivation on both carbon sources. However, the *malP* mutant changed its morphology in the course of cultivation with maltose. The change occurred at about the time point at which the growth rate dropped sharply (see above and Fig. 2c). Some cells then showed an irregular shape and an increased size (Fig. 4a). Single cells of *C. glutamicum ΔmalP* reached lengths of up to 10 µm. In the course of further cultivation on maltose, the number of irregularly shaped cells steadily increased; however, even 24 h after inoculation some regularly shaped cells were still visible (Fig. 4b).

As alterations in cell morphology might influence optical density determinations and also the viability of the cells, we determined the correlations of cell dry weight (dw) and OD 600 and of cell dw and the number of viable cells in the course of cultivation of *C. glutamicum* WT and *C. glutamicum ΔmalP* with either glucose (2%) or maltose (2%) as sole carbon source. However, both strains showed a linear correlation between cell dw and OD 600 (an OD 600 of 1 corresponded to about 0.35 ± 0.02 g dw l⁻¹) during exponential and early stationary growth phases with either or both carbon sources. A viability assay for *C. glutamicum* based on the different permeability of the cell membrane to the fluorescent stains SYTO 9 (green fluorescence) and propidium iodide (red fluorescence) was established. After incubation of *C. glutamicum* WT cells with a mixture of both fluorescent stains, living cells showed green fluorescence (Fig. 4c) and dead cells red fluorescence (Fig. 4d). Throughout cultivation of *C. glutamicum* WT with glucose or maltose and *C. glutamicum ΔmalP* with glucose, only green fluorescent cells and no red-stained cells were observed (data not shown). However, in the course of cultivation of *C. glutamicum ΔmalP* with maltose, the oversized cells showed red fluorescence (Fig. 4a), whereas...
the normally shaped cells showed green fluorescence. Samples from stationary-phase malP mutant cultures grown with maltose predominantly contained enlarged, red-fluorescent cells (Fig. 4b), indicating that most of the cells were dead. To corroborate these results, the viability of whole cultures of C. glutamicum ΔmalP was assayed by live-cell counting. Whereas in course of the cultivation on glucose the increase in the viable cell number (i.e. c.f.u.) correlated with the increase in cell dw, such a correlation was not observed for cultivation on maltose (Fig. 5). In other words, the ratio of the number of living cells per OD\textsubscript{600} unit was constant for C. glutamicum WT on either substrate and for C. glutamicum ΔmalP cultivated with glucose, but it decreased in the late-exponential and stationary phases in the C. glutamicum ΔmalP culture growing with maltose.

**Enzyme activities related to maltose metabolism**

In Gram-positive bacteria, permease-mediated maltose uptake is generally followed by utilization of the maltose by either maltose hydrolase or maltose phosphorylase and β-Pgm (Andersson & Radström, 2002; see Introduction). To test for the maltose utilization pathway in C. glutamicum, we cultivated this organism in minimal medium with maltose as the sole carbon source, prepared cell extracts from samples taken at mid-exponential growth phase (i.e. 6 h after inoculation) and at early stationary phase (i.e. 12 h after inoculation), and tested for maltose hydrolase, maltose phosphorylase and β-Pgm activities. None of these activities was detected in any of the cell extracts tested, and thus pathways generally assumed to be present in Gram-positive bacteria are unlikely to be present in C. glutamicum. In accordance with this finding, genes encoding the key enzymes of maltose metabolism in other Gram-positive bacteria have not been found in the C. glutamicum genome (Kalinowski et al., 2003).

The different growth phenotype of C. glutamicum ΔmalP in maltose medium suggested a role for MalP in maltose utilization in addition to its role in glycogen degradation.

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**Fig. 3.** TLC analysis of intracellular carbohydrates of C. glutamicum WT and C. glutamicum ΔmalP cells cultivated in minimal medium with maltose and harvested at the time points indicated. Lane S represents the separation of the standard sugars glucose (glc), maltose (m2), maltotriose (m3), maltoheptaose (m7) and glycogen (gly).

**Fig. 4.** Morphology and viability of C. glutamicum ΔmalP during cultivation with maltose as sole carbon source. (a) C. glutamicum ΔmalP 6 h cultivation in minimal medium (MM) with maltose; (b) C. glutamicum ΔmalP 24 h cultivation in MM with maltose. The morphology of living cells of C. glutamicum WT is shown in (c); dead cells of the WT are shown in (d). To assay for viability, WT cells cultivated in MM with maltose for 6 h were treated for 30 min with either saline (living cells) or 2-propanol (killed cells) before staining. For live/dead staining, see Methods.
Such a pathway would involve the formation of maltodextrins and therefore requires MalQ and in addition to MalP, a Glk, an α-Pgm and possibly also a MalZ (see Fig. 1). To test for this maltose utilization pathway in C. glutamicum, we first screened cell extracts for MalQ activity. For this purpose, glucose formation with maltose and maltotriose as substrates was measured in cell extracts, and we found that this activity was present in all samples. MalQ activity increased slightly from mid-exponential to early stationary growth phase from 0.26 to 0.33 U (mg protein)$^{-1}$. We also found glucose formation when incubating the cell extracts with maltodextrins of various lengths (from three to seven glucose residues) as substrates, indicating the presence of MalZ activity. To differentiate between MalQ and MalZ activity, we examined the maltodextrins formed in the course of the reaction catalysed by MalQ. As shown in Fig. 6, extracts from cells cultivated in medium with glucose or maltose were initially devoid of carbohydrate (lanes ce glc –, and ce malt –). After addition of maltose and maltotriose and incubation for 30 min, maltodextrins of various sizes were formed (Fig. 6, lanes ce glc + and ce malt +). Comparison of the respective spots revealed that the maltodextrins contained four to six glucose residues. In addition, a spot corresponding in size to glucose was detected. These results show the presence of a MalQ enzyme in C. glutamicum, able to generate various maltodextrins by transferring maltosyl residues from maltodextrins on maltose, thereby forming glucose.

We also detected the enzyme activities required for the processing of glucose 1-phosphate and glucose derived from the reactions of MalP and MalQ, respectively. High specific activities of α-Pgm, catalysing the reversible conversion of α-glucose 1-phosphate to glucose 6-phosphate, were present in extracts of cells cultivated in minimal medium containing either glucose or maltose. This activity was significantly higher in the early stationary [1.43 U (mg protein)$^{-1}$] than in the mid-exponential growth phase [0.68 U (mg protein)$^{-1}$]. Glk activity was also present in cell extracts of C. glutamicum, ranging from 0.04 U (mg protein)$^{-1}$ in the mid-exponential to 0.07 U (mg protein)$^{-1}$ in the early stationary phase.

Taken together, activities of MalQ, MalP, α-Pgm, Glk and probably also of MalZ were detected in C. glutamicum, and these findings indicate the presence of a pathway for maltose utilization similar to the one present in E. coli (Fig. 1).

**DISCUSSION**

We showed that C. glutamicum possesses MalP activity, that the enzyme is encoded by cg1479 (now designated malP) and that MalP of C. glutamicum is required for metabolism of intracellular maltodextrins formed in the course of both glycogen degradation and maltose metabolism. In addition to MalP, we observed GlgP activity in

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**Fig. 5.** Cell dw (lines) and c.f.u. (bars) of C. glutamicum ΔmalP during cultivation with glucose (light grey) or maltose (dark grey) as sole carbon source.

**Fig. 6.** TLC analysis of carbohydrates derived from maltose and maltotriose through the action of MalQ contained in preparations from C. glutamicum WT cells cultivated in media with either glucose (ce glc) or maltose (ce malt). The carbohydrates maltose and maltotriose (reference substances) are shown in lane C. The carbohydrate-free protein preparations are shown in (–) lanes. The products formed by carbohydrate-free protein preparations plus maltose and maltotriose are shown in (+) lanes. Lane S represents the separation of the standard sugars glucose (glc), maltose (m2), maltotriose (m3), maltoheptaose (m7) and glycogen (gly).
C. glutamicum, which was not affected in the ΔmalP strain. This observation indicates that in accordance with the glycogen degradation pathway in E. coli (Fig. 1), two different glucan phosphorylases, i.e. GlgP and MalP (Alonso-Casajus et al., 2006; Dauville et al., 2005), are required for efficient glycogen degradation in C. glutamicum. Indeed, we found evidence for a glgP gene, most probably encoding a protein with GlgP activity. Analysis of the deduced amino acid sequences of the two α-glucan phosphorylases of C. glutamicum revealed that they are phylogenetically distinct from each other (Fig. 7), belonging to different subgroups of α-glucan phosphorylases (Takahá et al., 2001). In the first subgroup, MalP of C. glutamicum is clustered with MalP and GlgP from E. coli and starch phosphorylase from Corynebacterium callunae. The second subgroup, containing GlgP of C. glutamicum, contains among others GlgP from Thermus aquaticus and AgpA from Thermotoga maritima. As both subgroups contain α-glucan phosphorylases with different substrate specificities, ranging from linear malto-dextrins to large, branched glucans such as glycogen or starch (Watson et al., 1999; Weinhaeusel et al., 1997; Bibel et al., 1998), predictions of specificities based on this classification are not possible. The phylogenetic analysis of the annotated

![Phylogenetic tree of α-glucan phosphorylases from various Corynebacterianeae](http://mic.sgmjournals.org)


The observed growth deficiencies of \textit{C. glutamicum} \textit{ΔmalP} during cultivation with maltose occur in conjunction with an altered cell shape. Disruption of \textit{malP} in \textit{E. coli} also alters the cell shape of the deletion mutant when cultivated with maltose (Schwartz, 1967). In accordance with the development of the maltodextrin content in \textit{C. glutamicum} \textit{ΔmalP} during cultivation with maltose, \textit{E. coli} \textit{ΔmalP} also accumulates maltodextrins, and this is thought to be the reason for the elongated cells of \textit{E. coli} \textit{ΔmalP}; however, the mechanism for this is not yet understood.

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$\alpha$-glucan phosphorylases from the \textit{Corynebacteriaeae} (Fig. 7) revealed that the number of annotated $\alpha$-glucan phosphorylases and the distribution among the subgroups vary among the species. The fact that most members of this suborder possess just one $\alpha$-glucan phosphorylase (e.g. \textit{Corynebacterium jeikeium, Corynebacterium efficiens, M. tuberculosis} H37Rv, \textit{Mycobacterium smegmatis}) suggests that, unlike in \textit{E. coli} and \textit{C. glutamicum}, $\alpha$-glucan phosphorylases of these \textit{Corynebacteriaeae} perform phosphorylative cleavage of both maltodextrins and glycogen, indicating an even closer entanglement of glycogen and maltose metabolism in these organisms than that described here for \textit{C. glutamicum}.

The interconnected pathways for maltose utilization and glycogen degradation in \textit{C. glutamicum} (depicted in Fig. 1) have been deduced from the detection of intracellular maltodextrins and from the demonstration of MalQ, MalP, $\alpha$-Pgm and Glk activities in \textit{C. glutamicum} cells. The presence of all these activities should be reflected by the presence of the respective genes in the genome. Apart from \textit{malP} (see above) and \textit{glk} (\textit{cg2399}) (Park \textit{et al.}, 2000), we identified \textit{cg2523} as probably encoding a MalQ and \textit{cg2800} as probably encoding $\alpha$-Pgm (Kalinskiow \textit{et al.}, 2003). The deduced amino acid sequences of these corynebacterial genes possess high identities (30–57%) to the respective proteins from \textit{E. coli}. The release of glucose from small maltodextrins suggested also the presence of MalZ; however, so far we have not been able to identify the corresponding \textit{C. glutamicum} gene from sequence comparisons. Since the presence of MalZ in \textit{C. glutamicum} could neither be clearly disproved nor confirmed, the pathway for maltose utilization in \textit{C. glutamicum} might be even more similar to the one found in the archaean \textit{Thermococcus litoralis} (Xavier \textit{et al.}, 1999) than to that in \textit{E. coli} (Boos & Shuman, 1998). The two pathways differ from each other, as MalZ is missing in \textit{Thermococcus litoralis} (Xavier \textit{et al.}, 1999).

Aside from enzyme patterns and the data from genome analysis, the phenotypes of \textit{C. glutamicum} \textit{ΔmalP} and of the \textit{glk} mutant (Park \textit{et al.}, 2000) of \textit{C. glutamicum} also support the proposed pathway for maltose utilization and thus corroborate the differences from other Gram-positive bacteria. When cultivated with maltose as the sole carbon source, \textit{C. glutamicum} \textit{ΔmalP} showed growth deficiencies in comparison with the WT and accumulated large amounts of intracellular maltodextrins in the course of growth. Due to the lack of MalP in the mutant, only one of the two glucose residues of maltose, i.e. that released by MalQ, could be further metabolized and consequently maltodextrins accumulate. For further utilization the glucose generated by the reaction of MalQ must be phosphorylated to glucose 6-phosphate by Glk. Since the \textit{glk} mutant of \textit{C. glutamicum} lacks Glk activity (Park \textit{et al.}, 2000), the glucose cannot be further metabolized. This might explain the growth deficiencies observed when cultivating the \textit{glk} deletion mutant of \textit{C. glutamicum} with maltose as the sole carbon source (Park \textit{et al.}, 2000).
Glucan phosphorylases of Corynebacterium glutamicum


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