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* $\Delta malP$ 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 $\Delta malP$ 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 (Glik) 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 Glik 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.

Received 22 August 2008

Revised 22 October 2008

Accepted 27 October 2008

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 *Corynebacterianae*, 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

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Abbreviations: cell dw, cell dry weight; WT, wild-type.

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 (GlcK) 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 phos-

phorylase 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 GlcK (Skarlatos & Dahl, 1998) or from β -glucose 1-phosphate by PgcM, a β -phosphoglucomutase, and glucose-1-phosphate dismutase (Mesak & Dahl, 2000). 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;

Fig. 1. Schematic diagram of pathways for glycogen degradation and maltose/maltodextrin metabolism in *E. coli*. Abbreviations: GlgP, glycogen phosphorylase; GlgX, debranching enzyme; GlcK, glucokinase; KDPG pathway, 2-keto-3-deoxy-6-phosphogluconate pathway; MalP, maltodextrin phosphorylase; MalQ, 4- α -glucanotransferase; MalZ, maltodextrin glucosidase; α -Pgm, α -phosphoglucomutase; pl-dextrins, phosphorylase-limited dextrins; PP pathway, pentose phosphate pathway.

Tzvetkov *et al.*, 2003; Woodruff *et al.*, 2004). Trehalose in these bacteria serves as a stress protection compound and is a prerequisite for the production of mycolates, major and structurally important constituents of the cell envelope of *Corynebacteriaceae* (Wolf *et al.*, 2003; Tropis *et al.*, 2005).

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 GlgP 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* DH5 α (Hanahan, 1983) and *C. glutamicum* wild-type (WT) (ATCC 13032, American type Culture Collection). Plasmid pK19mobsacB (Schäfer *et al.*, 1994) was employed for construction of *C. glutamicum* Δ malP, and plasmid pEKEx2 (Eikmanns *et al.*, 1991a) for construction of pEKEx2-glgP2. *E. coli* and all pre-cultures of *C. glutamicum* were grown aerobically in TY complex medium (Sambrook *et al.*, 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 (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 'fedbatch-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 μ m, Sigma) and subjected five times for 30 s to mechanical disruption with a RiboLyser (Hybaid) 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 bicinchoninic acid protein assay reagent kit (Pierce) with BSA as the standard.

4- α -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 (GlcK) 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 & Radström (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 hydrolase 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 maltoheptaose 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 maltoheptaose and the reduction of NADP was monitored. For the determination of the substrate specificity, experiments were performed with maltohexaose, 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 maltoheptaose as the substrate.

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 *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 μ g ml⁻¹). Electroporation 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 cyler (Biotron). Deoxynucleoside 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 pK19mobsacB. *malP*-specific DNA fragments were generated using the primer pairs MalPDelFor1 (5'-CGGAATTCGGATGCTCAGGACCTTATTG-3', *Eco*RI site underlined) and MalPDelRev1 (5'-GGAATGGAGTATGGAAGTTGGCCATATCCGGCATGCTTAG-3', crossover overlap in italic type), and MalPDelFor2 (5'-CCAACTTCCATACTCCATTCCGCCATATCCCGCTCAAC-3', crossover overlap in italic type) and MalPDelRev2 (5'-CGGGATCCAAATTCGGCTTGCACTAGAC-3', *Bam*HI site underlined). Fragment 1 covers the region 769–90 bp upstream of the *malP* gene; fragment 2 covers the region 228–855 bp downstream of the stop codon. The two fragments were purified, mixed in equal amounts and subjected to crossover PCR using primers MalPDelFor1 and MalPDelRev2. Using the PCR-generated 5' *Eco*RI and 3' *Bam*HI restriction sites of the fusion product, the construct was ligated into pK19mobsacB and transformed into *E. coli*. The recombinant plasmid was isolated from *E. coli* and electroporated into *C. glutamicum* WT. By application of the method described by

Schäfer *et al.* (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 *Bam*HI-restricted and size-fractionated chromosomal DNA from *C. glutamicum* WT and the potential deletion mutant *C. glutamicum* Δ *malP*. 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* Δ *malP*. 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 pEKEx2-*glgP* was constructed. For this purpose, *glgP* (*cg2289*) was amplified by PCR using the primers OE*glgP*-for (5'-ACGCGTCGACTTCCTGGTAAGCAGGAACCC-3', *Sal*I site underlined) and OE*glgP*-rev (5'-CGGAATTCGGTAAAGCTAGTTTCGCC-3', *Eco*RI site underlined). Using the PCR-generated 5' *Sal*I and 3' *Eco*RI restriction sites of the PCR product, the construct was ligated into pEKEx2 and transformed into *E. coli*. The recombinant plasmid was isolated from *E. coli* and electroporated into *C. glutamicum* WT, resulting in *C. glutamicum* (pEKEx2-*glgP*). For induction of the *glgP* gene, 0.5 mM IPTG was added to the culture.

Microscopic imaging. For phase-contrast and fluorescence microscopy, 2 μ l 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 $\times$ /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.

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-GI 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.

RESULTS

Activities of GlgP and MalP in *C. glutamicum*

To complete our previous investigations of glycogen degradation in *C. glutamicum* (Seibold & Eikmanns,

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 α -glucan phosphorylases from *Thermus aquaticus* and *Thermotoga maritima*, respectively (Takaha *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* Δ *malP*.

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-*glgP*), which expresses *cg2289* under control of the *tac* promoter. Indeed, the specific GlgP activity in cell extracts of *C. glutamicum* (pEKEx2-*glgP*) 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* Δ *malP*

To test for physiological consequences of *malP* inactivation in *C. glutamicum*, we analysed the growth and glycogen content of *C. glutamicum* Δ *malP* 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* Δ *malP* 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* Δ *malP* (Fig. 2b). When cultivated with 2 % maltose, the growth behaviour of *C. glutamicum* Δ *malP* 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 OD₆₀₀ was slightly lower in comparison with the WT strain. The slower growth of *C. glutamicum* Δ *malP* was in accordance with the CO₂ 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* Δ *malP*, and remained roughly constant for the WT until the substrate was consumed (Fig. 2c). In contrast, maltose utilization of the Δ *malP* 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* Δ *malP* 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

Fig. 2. Growth (circles), substrate concentrations (triangles) and glycogen content (squares) of *C. glutamicum* WT (filled symbols) and *C. glutamicum* $\Delta malP$ (open symbols) during cultivation in minimal medium with 2% glucose [(a) growth and substrate concentrations; (b) glycogen content] or with 2% maltose [(c) growth and substrate concentrations; (d) glycogen content] as carbon source.

and 48 h after inoculation. Samples from *C. glutamicum* $\Delta malP$ 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* $\Delta malP$

Microscopic analysis revealed no significant differences in cell shape for *C. glutamicum* $\Delta malP$ 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* $\Delta malP$ reached lengths of up to 10 μ m. 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 OD₆₀₀ and of cell dw and the number of viable cells in the course of cultivation of *C. glutamicum* WT and *C. glutamicum* $\Delta malP$ with either glucose (2%) or maltose (2%) as sole carbon source. However, both strains showed a linear correlation between cell dw and OD₆₀₀ (an OD₆₀₀ 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* $\Delta malP$ 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* $\Delta malP$ with maltose, the oversized cells showed red fluorescence (Fig. 4a), whereas

Fig. 3. TLC analysis of intracellular carbohydrates of *C. glutamicum* WT and *C. glutamicum* $\Delta malP$ 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).

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* $\Delta malP$ 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 OD₆₀₀ unit was constant for *C. glutamicum* WT on either substrate and for *C. glutamicum* $\Delta malP$ cultivated with glucose, but it decreased in the late-exponential and stationary phases in the *C. glutamicum* $\Delta malP$ 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

Fig. 4. Morphology and viability of *C. glutamicum* $\Delta malP$ during cultivation with maltose as sole carbon source. (a) *C. glutamicum* $\Delta malP$ 6 h cultivation in minimal medium (MM) with maltose; (b) *C. glutamicum* $\Delta malP$ 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.

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* $\Delta malP$ in maltose medium suggested a role for MalP in maltose utilization in addition to its role in glycogen degradation.

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

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

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).

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

C. glutamicum, which was not affected in the $\Delta malP$ 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; Dauvillee *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

(Takaha *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 maltodextrins to large, branched glucans such as glycogen or starch (Watson *et al.*, 1999; Weinhäusel *et al.*, 1997; Bibel *et al.*, 1998), predictions of specificities based on this classification are not possible. The phylogenetic analysis of the annotated

Fig. 7. Phylogenetic tree of α -glucan phosphorylases from various *Corynebacteriaceae* showing the distribution into the two subgroups (Takaha *et al.*, 2001). Key members [MalP and GlgP of *E. coli* and glucan phosphorylase (GP) from *Thermus aquaticus* and *Thermotoga maritima*] of each subgroup are underlined; MalP and GlgP of the *C. glutamicum* type strain (13032) are indicated by bold type. The tree is based on multiple sequence alignments obtained by using CLUSTAL W and visualized using Dendroscope (Huson *et al.*, 2007). The branch lengths are proportional to the sequence divergence; the scale bar represents 0.1 amino acid replacements per site. The NCBI-GI accession numbers of the glucan phosphorylases shown are: 21464507, starch phosphorylase (SP), *Corynebacterium callunae*; 68536348, GP, *C. jeikeium* K411; 145295449, CgR_1383, *C. glutamicum* R; 145295448, CgR_1382, *C. glutamicum* R; 38234127, GP, *C. diphtheriae* NCTC 13129; 169628942, GP1, *Mycobacterium abscessus*; 120402204, GP1, *Mycobacterium vanbaalenii* PYR-1; 145225720, GP1, *Mycobacterium gilvum* PYR-GCK; 183982578, GP1, *Mycobacterium marinum* M; 145296049, CgR_1971, *C. glutamicum* R; 25028543, GP, *C. efficiens* YS-314; 120405254, GP2, *M. vanbaalenii* PYR-1; 145222933, GP2, *M. gilvum* PYR-GCK; 126436421, GP, *Mycobacterium* sp. JLS; 54023047, GP, *Nocardia farcinica* IFM 10152; 169628560, GP2, *M. abscessus*; 118470308, GP, *M. smegmatis* mc²155; 183984041, GP2, *M. marinum* M; 118619142, GP, *Mycobacterium ulcerans* Agy99; 111018451, GP, *Rhodococcus* sp. RHA1; 15608468, GP, *M. tuberculosis* H37Rv; 31792524, GP, *Mycobacterium bovis* AF2122/97; 118465028, GP, *M. avium* 104; 81254715, GP1, *M. tuberculosis* C; 81254714, GP2, *M. tuberculosis* C.

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

The interconnected pathways for maltose utilization and glycogen degradation in *C. glutamicum* (depicted in Fig. 1) have been deduced from the detection of intracellular maltodextrins and from the demonstration of MalQ, MalP, α -Pgm and Glk activities in *C. glutamicum* cells. The presence of all these activities should be reflected by the presence of the respective genes in the genome. Apart from *malP* (see above) and *glk* (*cg2399*) (Park *et al.*, 2000), we identified *cg2523* as probably encoding a MalQ and *cg2800* as probably encoding α -Pgm (Kalinowski *et al.*, 2003). The deduced amino acid sequences of these corynebacterial genes possess high identities (30–57%) to the respective proteins from *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 *C. glutamicum* gene from sequence comparisons. Since the presence of MalZ in *C. glutamicum* could neither be clearly disproved nor confirmed, the pathway for maltose utilization in *C. glutamicum* might be even more similar to the one found in the archaeon *Thermococcus litoralis* (Xavier *et al.*, 1999) than to that in *E. coli* (Boos & Shuman, 1998). The two pathways differ from each other, as MalZ is missing in *Thermococcus litoralis* (Xavier *et al.*, 1999).

Aside from enzyme patterns and the data from genome analysis, the phenotypes of *C. glutamicum* Δ malP and of the *glk* mutant (Park *et al.*, 2000) of *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, *C. glutamicum* Δ malP 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 *glk* mutant of *C. glutamicum* lacks Glk activity (Park *et al.*, 2000), the glucose cannot be further metabolized. This might explain the growth deficiencies observed when cultivating the *glk* deletion mutant of *C. glutamicum* with maltose as the sole carbon source (Park *et al.*, 2000).

The observed growth deficiencies of *C. glutamicum* Δ malP during cultivation with maltose occur in conjunction with an altered cell shape. Disruption of *malP* in *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 *C. glutamicum* Δ malP during cultivation with maltose, *E. coli* Δ malP also accumulates maltodextrins, and this is thought to be the reason for the elongated cells of *E. coli* Δ malP; however, the mechanism for this is not yet understood.

ACKNOWLEDGEMENTS

The authors thank Petra Dangel and Eva Glees for excellent technical assistance. The support of Evonik Degussa AG, Halle-Künsebeck, is gratefully acknowledged.

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Edited by: M. Hecker