Transcription of malP is subject to phosphotransferase system-dependent regulation in Corynebacterium glutamicum

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The Gram-positive Corynebacterium glutamicum co-metabolizes most carbon sources such as the phosphotransferase system (PTS) sugar glucose and the non-PTS sugar maltose. Maltose is taken up via the ABC-transporter MusEFGK, and is further metabolized to glucose phosphate by amylomaltase MalQ, maltodextrin phosphorylase MalP, glucokinase Glk and phosphoglucomutase Pgm. Surprisingly, growth of C. glutamicum strains lacking the general PTS components EI or HPr was strongly impaired on the non-PTS sugar maltose. Complementation experiments showed that a functional PTS phosphorelay is required for optimal growth of C. glutamicum on maltose, implying its involvement in the control of maltose metabolism and/or uptake. To identify the target of this PTS-dependent control, transport measurements with 14C-labelled maltose, Northern blot analyses and enzyme assays were performed. The activities of the maltose transporter and enzymes MalQ, Pgm and Glk were not decreased in PTS-deficient C. glutamicum strains, which was corroborated by comparable transcript amounts of musE, musK and musG, as well as of malQ, in C. glutamicum ΔptsH and WT. By contrast, MalP activity was significantly reduced and only residual amounts of malP transcripts were detected in C. glutamicum ΔptsH when compared to WT. Promoter activity assays with the malP promoter in C. glutamicum ΔptsH and WT confirmed that malP transcription is reduced in the PTS-deficient strain. Taken together, we show here for what is to the best of our knowledge the first time a regulatory function of the PTS in C. glutamicum and identify malP transcription as its target.

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INTRODUCTION

Bacterial phosphoenolpyruvate:carbohydrate phosphotransferase systems (PTSs) catalyse uptake and concomitant phosphorylation of their cognate substrates, being mostly sugars and sugar derivatives (Postma et al., 1993). The PTS is usually composed of the two general proteins, enzyme I and HPr, and three substrate-specific proteins or domains, EIIA, EIIB and the membrane-spanning EIIC. The phosphorylation cascade required for transport and phosphorylation of substrates starts with the autophosphorylation of EI dimers at a conserved histidyl residue at the expense of phosphoenolpyruvate (Alpert et al., 1985; Ginsburg & Peterkofsky, 2002; Venditti & Clore, 2012). After phosphorylation, the resulting EI~P dimer dissociates and subsequently donates the phosphate group to HPr at the N1 position of the highly conserved histidyl residue (e.g. His-15 in Escherichia coli HPr) (Waygood, 1998). Subsequently HPr~P phosphorylates a histidyl residue of EIIA (Dörschug et al., 1984; Koch et al., 1996). Finally EIIA~P phosphorylates a histidyl or cysteinyl residue of EIIB, which itself phosphorylates the substrate.

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Abbreviations: CCR, carbon catabolite repression; PTS, phosphotransferase system

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transported via the membrane-spanning component EIIC (Cao et al., 2011; Chartrier et al., 1997; Pas & Robillard, 1988). All reactions within the PTS are reversible with the exception of the final step, namely substrate phosphorylation. By this means, the phosphorylation state of PTS components reflects, beside carbohydrate availability, also the physiological state of the cell (Görke & Stülke, 2008). Therefore, PTSs often possess, in addition to their catalytic function as importers of sugars and/or sugar derivatives, tasks within the control of diverse cellular functions, e.g. carbon metabolism, nitrogen metabolism, chemotaxis, biofilm formation and/or virulence (Deutscher et al., 2014; Gabor et al., 2011). To adjust metabolism to nutrient availability, expression of enzymes for transport and catabolism of less favourable substrates is often prevented in the presence of the preferred carbon source. This regulatory phenomenon, named carbon catabolite repression (CCR), requires in both Gram-negative and Gram-positive bacteria various inputs by the PTS (Deutscher, 2008; Görke & Stülke, 2008).

Inducer exclusion, the major CCR mechanism in E. coli and Salmonella enterica serovar Typhimurium, inhibits the utilization of, for example, lactose, glycerol and maltose in the presence of the PTS substrate glucose and requires EIIAGlc (enzyme IIA of the glucose PTS) as the central player (Deutscher et al., 2014; Görke & Stülke, 2008; Inada et al., 1996). EIIAGlc becomes dephosphorylated during the transport of readily metabolizable substrate glucose (Hogema et al., 1998). Unphosphorylated EIIAGlc in turn binds and inactivates metabolic enzymes (e.g. glyceraldehyde-3-phosphate dehydrogenase) and non-PTS transporters such as the lactose permease LacY and the maltose uptake system MalFGK2E (Dean et al., 1990; Hurley et al., 1993; Osumi & Saier, 1982). By this means, disruption of the PTS phosphorelay by deletion of ptsI or ptsH leads to the inhibition of maltose uptake in E. coli and S. enterica serovar Typhimurium and, thus, exclusion of the inducer (Bao & Duong, 2013; Blüschke et al., 2006; Chen et al., 2013; Saier & Roseman, 1976a, b; Simoni et al., 1976). Furthermore, PTS components are also directly involved, by protein–protein interactions, in the control of chemotaxis (unphosphorylated EI inhibits CheA autophosphorylation), glycogen metabolism (dephosphorylated HPr binds and activates the glycogen phosphorylase) and σ^70-dependent transcription (in stationary-phase cells HPr forms a tight complex with the anti-σ factor Rsd and thereby inhibits complex formation between Rsd and σ^70) (Lux et al., 1995; Park et al., 2013; Seok et al., 1997).

CCR in low-G+C Gram-positives like Bacillus subtilis also involves the PTS components (Deutscher et al., 2014; Görke & Stülke, 2008). In these bacteria, HPr can get also phosphorylated at Ser-46 by the bifunctional HPr kinase/phosphorylase HPrK. The product P-Ser46-HPr has a significantly lower affinity to EI than free HPr (Deutscher et al., 1984); thus, PTS transport gets limited by a feedback mechanism. In the presence of readily metabolizable substrates, HPr is not phosphorylated at His-15 and concentrations of FBP and ATP increase because of the high glycolytic activity. These conditions favour the HPrK kinase activity resulting in phosphorylation of HPr at Ser-46, which in turn binds to and activates the catabolite control protein A (CcpA) (Deutscher & Saier, 1983; Poncet et al., 2004; Ramström et al., 2003). The resulting CcpA and P-Ser46-HPr complex binds to DNA at catabolite responsive elements (CREs) (Schumacher et al., 2004, 2011) and by this means controls activity of roughly 300 target operons in B. subtilis (Fujita, 2009; Marcinia et al., 2012). In the firmicutes Lactococcus lactis and Lactobacillus casei, P-Ser46-HPr triggers inducer exclusion through the inhibition of maltose uptake (Monedero et al., 2001, 2008). The PTS also controls CcpA-independent catabolite control in Gram-positives. In the absence of a readily metabolizable carbon source HPr~P phosphorolyases transcription regulators, all of which contain the PTS regulatory domain (PRD) and enhance expression of proteins necessary for utilization of secondary carbon sources such as levan and β-glucosides in B. subtilis (Débarbouille et al., 1991; Lindner et al., 1999; Martin-Veralba et al., 1998; Schnetz et al., 1996).

The high G+C, Gram-positive Corynebacterium glutamicum serves as a workhorse in industrial amino acid production and has been genetically engineered as a versatile platform for the production of several bulk chemicals (Eggeling & Bott, 2005; Wendisch, 2014). In contrast to other bacteria, C. glutamicum efficiently co-utilizes most carbon sources (Arndt & Eikmanns, 2008) and does not typically form by-products indicating overflow metabolism during fully aerobic cultivations (Koch-Koerfges et al., 2012; Paczia et al., 2012). From these observations, the existence of elaborate mechanisms for the control and coordination of carbon metabolism can be deduced and indeed complex networks of transcriptional regulators have been identified in C. glutamicum (Bott & Brocker, 2012; Schröder & Tauch, 2010; Teramoto et al., 2011). Surprisingly, the role of PTS components in the control of metabolism has hitherto not been elucidated in C. glutamicum. The PTS was shown to be the major system of glucose-utilization, and essential for fructose and sucrose utilization in C. glutamicum (Lindner et al., 2011; Moon et al., 2005). The PTS components enzyme I (EI), HPr and four enzyme II perases were identified and analysed (Moon et al., 2005; Parche et al., 2001). Of the latter, the ptsF-encoded EIIBCA^Glc catalyses uptake and phosphorylation of both glucose and glucosamine, the ptsL^Fru and ptsS-encoded permeses EIABC^Fru and EIIBCA^Suc fructose and sucrose uptake, respectively (Moon et al., 2005; Uhde et al., 2013). Albeit a serine residue is present at position 46 in C. glutamicum HPr (Fig. S1, available in the online Supplementary Material), neither a gene for nor activity of HPrK was detected in C. glutamicum (Kaliniowski et al., 2003; Parche et al., 2001). Because of the poor growth on maltose of the EI-deficient C. glutamicum mutant strain GSJ125, it was concluded that maltose might be a PTS substrate or the...
maltose uptake system regulated by the PTS via inducer exclusion (Parche et al., 2001). However, we recently showed that maltose uptake exclusively proceeds in *C. glutamicum* via the ABC-transporter MusEFGK$_2$I (Henrich et al., 2013). As depicted in Fig. 1(a), maltose is then metabolized by the concomitant action of amylomaltase MalQ, maltodextrin phosphorylase MalP, the glucokinases Glk and Ppgk, and phosphoglucomutase Pgm (Clermont et al., 2015; Lindner et al., 2010; Seibold et al., 2009; Seibold & Eikmanns, 2013); genes for maltose utilization are not clustered within the *C. glutamicum* genome (Fig. 1b; Kalinowski et al., 2003). As EI is not directly involved in maltose metabolism, but its absence resulted in poor growth with maltose, a regulatory function of PTS elements for the control of maltose metabolism may be assumed.

Here, we describe the investigation of the roles of EI and HPr in maltose metabolism in *C. glutamicum* and show that indeed both EI and HPr are essential for efficient growth of *C. glutamicum* strains on maltose, although maltose uptake required neither EI nor HPr. Instead, we show MalP activity and malP transcription to be dependent on a functional PTS; thus, demonstrating for what is to the best of our knowledge the first time a regulatory function of the PTS in *C. glutamicum*.

**METHODS**

**Bacterial strains, media and culture conditions.** Bacteria and plasmids used in this study are listed in Table 1. *E. coli* and all pre-cultures of *C. glutamicum* were grown aerobically in TY complex medium (Sambrook et al., 2001) at 37 and 30 °C, respectively, as 50 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 twice with 0.9 % (w/v) NaCl and then inoculated into CGC minimal medium (Eikmanns et al., 1991b) containing maltose, glucose or sucrose as carbon sources, as indicated in Results. When appropriate, kanamycin (50 μg ml$^{-1}$) and IPTG (up to 250 μM) were added to

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**Fig. 1.** (a) Schematic diagram of the pathway for maltose metabolism in *C. glutamicum*. Glk, Glucokinase; MalP, maltodextrin phosphorylase; MalQ, 4-α-glucanotransferase; MusEFGK$_2$I, ABC-transporter for maltose; Pgm, α-phosphoglucomutase; Ppgk, polyphosphate-dependent glucokinase. (b) Genetic organization of the genes for maltose utilization (grey arrows) in *C. glutamicum*, neighbouring ORFs not related to maltose metabolism are depicted as white arrows; the annotation and ORF numbers are given according to the genome sequence published by Kalinowski et al. (2003). The white arrows annotated—–with hyphens indicate ORFs with unknown functions.
the media. Growth of E. coli and of C. glutamicum was followed by measuring the optical density at 600 nm.

**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 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-agar plates containing kanamycin (25 μg ml⁻¹). Electroporation of E. coli was performed according to the method of Dower et al. (1988). All enzymes used were obtained from New England Biolabs and used according to the instructions of the manufacturer. PCR experiments were performed in a TProfessional thermocycler (Biometra). Deoxyribonucleotide triphosphates were obtained from Bio-Budget, oligonucleotides (primers) from Eurofins MWG Operon. Cycling times and temperatures were chosen according to fragment length and primer constitution. PCR products were separated in agarose gels and purified using the Nucleospin extract II kit (Macherey–Nagel).

**Construction of expression vectors.** For IPTG-inducible overexpression vector pEKE2x was used. Genes were amplified via PCR from genomic DNA of C. glutamicum ATCC 13032 using the oligonucleotide primers listed in Table S1. The resulting PCR products were introduced into the cloning vector pJET1.2 (MBI Fermentas) according to the manufacturer’s instructions. Primer-attached restriction sites of the PCR products (indicated in Table S1) were used to excise the inserts, the resulting fragments were ligated into the plasmid pEKE2x (digested with appropriate enzymes) and used to transform E. coli DH5α. The resulting plasmids were isolated and the nucleotide sequences confirmed by sequencing (GATC Biotech).

**Construction of the deletion mutants.** For in-frame deletion of ptsI, flanking regions of the gene of roughly 800 bp were amplified by using primer pairs ptsI/Del-A and ptsI/Del-B and ptsI/Del-C and ptsI/Del-D. The PCR products were fused via the complementary artificial overhangs provided in primers ptsI/Del-B and ptsI/Del-C in a PCR using both of the PCR products together with primers ptsI/Del-A and ptsI/Del-D. The resulting approximately 1600 bp long PCR product was cloned blunt end into SmaI restricted vector pK19mobsacB resulting in pK19mobsacBΔptsl. The deletion of ptsI was verified using the primers ΔptsI/Ver-fw and ΔptsI/Ver-rv, resulting in a 3346 bp fragment for WT and a 1692 bp fragment for the deletion mutant. In-frame deletion of ptsG in C. glutamicum ΔptsH was performed as described using the plasmid pK19mobsacBΔptsG (Henrich et al., 2013). The deletion of ptsG was verified using the primers ΔptsG_test-for and ΔptsG_test-rev, in a 3583 bp fragment for WT and a 1867 bp fragment for the deletion mutant. Deletion of ptsH was also confirmed by PCR using the primer pair ΔptsH_test-for and ΔptsH_test-rev, in an 805 bp fragment for WT and a 616 bp fragment for the deletion mutant.

**Construction of a reporter gene fusion with the promoter of malP.** The corynebacterial promoter-probe vector pEPI was used to construct a transcriptional fusion of the malP promoter to the promoterless gfp gene. Therefore, the malP promoter region was amplified by PCR with the primers PmalP-for and PmalP-rev (Table S1) from genomic DNA of C. glutamicum WT. The 322 bp PCR product, covering the region from 257 bp upstream to 50 bp

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

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant characteristics</th>
<th>Reference/source</th>
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<tr>
<td><strong>Strains</strong></td>
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<tr>
<td>C. glutamicum</td>
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<tr>
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<td>WT strain ATCC 13032</td>
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<td>Henrich et al. (2013)</td>
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<td>C. glutamicum WT with deletion of ptsH (cg2121)</td>
<td>Lindner et al. (2011)</td>
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<td>This work</td>
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<tr>
<td>ΔptsI</td>
<td>C. glutamicum WT with deletion of ptsI (cg2117)</td>
<td>This work</td>
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<tr>
<td>HSM</td>
<td>Suppressor mutant of C. glutamicum ΔptsH isolated after consecutive cultivations on maltose</td>
<td>This work</td>
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<td><strong>Plasmids</strong></td>
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<td>pK19mobsacB</td>
<td>KmR, mobilizable E. coli vector for the construction of insertion and deletion mutants in C. glutamicum (oriV, sacb, lacZα)</td>
<td>Schäfer et al. (1994)</td>
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<tr>
<td>pK19mobsacBΔptsG</td>
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<td>Promoter probe vector carrying the promoterless gfp gene, KmR</td>
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<td>Expression vector, ptac, lacI, KmR</td>
<td>Eikmanns et al. (1991a)</td>
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<td>pEKE2x carrying ptsH gene with altered codon for amino acid exchange H15A and sequence for C-terminal FLAG-tag</td>
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downstream of the malIP ATG translational start codon, was digested with XbaI and BamHI and ligated into the multiple cloning site of pEPRI and BamHI and ligation into the multiple cloning site of pEPRI-PRmalIP. Promoter activities of C. glutamicum strains carrying pEPRI-PRmalIP were measured by determining the GFP fluorescence in relation to the optical density measured at 600 nm using an Infinite M200 plate reader (Tecan).

**Site-directed mutagenesis.** Exchange of the HPr His-15 codon to an alanine codon in pEKE2-ptsH was performed using the QuikChange lighting site-directed mutagenesis kit (Agilent Technologies). PCR with pEKE2-ptsH as the template and the two complementary oligonucleotides ptsH-H15A_for and ptsH-H15A_rev, which contain the desired point mutations (Table S1), was performed according to the manufacturer’s protocol. Non-mutated, methylated parental plasmid DNA was degraded by DpnI digestion; the obtained plasmids carrying the desired mutation were used to transform E. coli DH5α. The resulting plasmid pEKE2-ptsH-H15A was isolated and the nucleotide sequence confirmed by sequencing (GATC Biotech).

**Protein analysis.** Protein concentrations were determined using the Roti-Nanoquant kit (Roth) with BSA as the standard. SDS-PAGE was performed according to Laemmli (1970). Standard loading buffer (4 × 100 μL) contained 8 % (w/v) SDS, 20 % (v/v) glycerol, 10 mM EDTA, 100 mM Tris/HCl, pH 6.8, 2 % (v/v) β-mercaptoethanol and 1 mg bromophenol blue ml⁻¹. Western blot experiments for detection of the FLAG-tagged HPr-FLAG and HPr-H14A-FLAG proteins in cell extracts were performed using the anti-FLAG M2 mAb and alkaline phosphatase-conjugated anti-mouse antibody (Sigma-Aldrich).

**Enzyme assays.** Activities of MalP, Pgm, glucokinase, pyruvate kinase (Pyk) and MalQ 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 as described elsewhere (Clermont et al., 2015; Netzer et al., 2004; Seibold et al., 2009; Seibold & Eikmanns, 2013).

**RNA techniques.** Isolation of total RNA from C. glutamicum cells was performed using a Nucleospin RNAII kit (Macherey-Nagel) as described by Wolf et al. (2003). For Northern (RNA) hybridization, DIG-11-DUTP-labelled gene-specific antisense RNA probes were prepared from PCR products (generated with the oligonucleotides listed in Table S1) carrying the T7 promoter by in vitro transcription (1 h, 37 °C) using T7 RNA polymerase (MBI Fermentas). Slot blot experiments, detection and signal quantification were performed as described elsewhere (Lindner et al., 2013; Möker et al., 2004).

**[¹⁴C]Maltose uptake studies.** Maltose uptake studies were performed essentially as described by Henrich et al. (2013). In detail, C. glutamicum cells were grown in the media indicated in the text to mid-exponential growth phase, harvested by centrifugation, washed twice with ice-cold CGC medium, suspended to an OD₆₀₀ of 2 with CGC medium and stored on ice until the measurement was carried out. Before the transport assay, cells were incubated for 3 min at 30 °C; the reaction was started by addition of 50 μM [¹⁴C]maltose [specific activity 679 mCi μmol⁻¹ (25.123 GBq μmol⁻¹); Amersham]. At given time intervals (15, 30, 45, 60 and 120 s), 200 μL samples were filtered through glass fibre filters (Typ F; Millipore) and washed twice with 2.5 ml of 100 mM LiCl. The radioactivity of the samples was determined using scintillation fluid (Rotiszinth; Roth) and a scintillation counter (LS 6500; Beckmann).

**Computational analysis.** Database searches were carried out by using BLAST (Altschul et al., 1990) and protein sequences were analysed using CLUSTAL W (Thompson et al., 1994). The following National Center for Biotechnology Information GI accession numbers for protein sequences were retrieved from the KEGG database (Kanehisa et al., 2008): 16130341 – E. coli HPr, 16078454 – B. subtilis HPr, 62390777 – C. glutamicum HPr.

**RESULTS**

A functional PTS is required for efficient maltose utilization

In order to test the impact of PTS deficiency on maltose utilization, the ptsI deletion mutant C. glutamicum ΔptsI was created. As expected from the results of other studies (Moon et al., 2005; Parche et al., 2001), C. glutamicum ΔptsI was not able to use the PTS substrate sucrose for growth in control experiments (Fig. S2). Growth of C. glutamicum ΔptsI in minimal medium containing maltose was slowed down to a growth rate of 0.06 ± 0.01 h⁻¹, which is only about 15 % of the growth rate of 0.36 ± 0.02 h⁻¹ observed for the parental strain C. glutamicum WT in cultivations on maltose (Fig. 2). However, the final OD₆₀₀ reached after 24 h cultivation in minimal medium with 1 % (w/v) maltose was nearly identical for both C. glutamicum ΔptsI and WT (a final OD₆₀₀ of 19.4 ± 3.3 and 18.7 ± 2.6 was observed, respectively). Growth on maltose, as well as on sucrose, was fully restored to WT levels upon ectopic expression of ptsI in the strain.

**Fig. 2.** Growth in minimal medium with 1 % (w/v) maltose of C. glutamicum WT (○), ΔptsI (□), ΔptsI (pEKE2) (▲) and ΔptsI (pEKE2-ptsI) (Δ). Three independent cultivations were performed; data from one representative experiment are shown. Plasmids were induced by the addition of 100 μM IPTG.
C. glutamicum ∆ptsI (pEKE2-ptsI), whereas growth of the empty vector control strain C. glutamicum ∆ptsI (pEKE2) remained slow on maltose and absent on sucrose (Figs 2 and S2).

To further study the importance of the PTS for maltose utilization in C. glutamicum, the growth of the ptsH-deletion mutant C. glutamicum ∆ptsH on maltose was analysed also. When compared to C. glutamicum WT, the growth of C. glutamicum ∆ptsH in minimal medium with 1 % (w/v) maltose was slowed to a growth rate of 0.07 ± 0.02 h⁻¹ (Fig. 3a), which is comparable to the growth rate described above for C. glutamicum ∆ptsI. As expected, C. glutamicum ∆ptsH did not grow in control experiments on sucrose as the sole carbon substrate (Fig. S3a). Growth on maltose, as well as on sucrose, was fully restored to WT levels upon ectopic expression of a FLAG-tagged HPr-variant in the strain C. glutamicum ∆ptsH (pEKE2-ptsH), whereas growth of the empty vector control strain C. glutamicum ∆ptsH (pEKE2) remained slow on maltose and absent on sucrose (Figs 3b and S3b). As both C. glutamicum ∆ptsI and ∆ptsH showed poor growth on maltose, the role of HPr phosphorylation at His-15 for maltose utilization was analysed. Using site-directed mutagenesis the plasmid pEKE2-ptsH-H15A was generated, which encodes an HPr-H15A variant. In HPr-H15A, the His-15 residue was exchanged to alanine; thus, EI-dependent phosphorylation of Hpr should be abolished. The plasmid pEKE2-ptsH-H15A was introduced into C. glutamicum ∆ptsH and, as expected, the resulting strain ∆ptsH (pEKE2-ptsH-H15A) was not able to grow with sucrose as the sole carbon source, as PTS phosphorylation and phosphorylation of PTS substrates requires HPr phosphorylation at His-15 (Fig. S3b). Growth of C. glutamicum ∆ptsH (pEKE2-ptsH-H15A) on maltose proceeded as slowly as reported above for the parental strain ∆ptsH; a growth rate of 0.06 ± 0.02 h⁻¹ was determined for C. ∆ptsH (pEKE2-ptsH-H15A) (Fig. 3b). In Western blot analysis with antibodies against the FLAG epitope present in the HPr variants encoded by pEKE2-ptsH, as well as pEKE2-ptsH-H15A, one single band with an apparent molecular mass of 15 kDa was detected in extracts of ∆ptsH (pEKE2-ptsH) cells from exponentially growing cultivations on maltose (4 h after inoculation), whereas two bands with apparent molecular masses of 15 and 14 kDa were detected in extracts of ∆ptsH (pEKE2-ptsH) samples from the stationary growth phase (24 h after inoculation) (Fig. 3c). One single band with an apparent molecular mass of 15 kDa was detected in extracts of C. glutamicum ∆ptsH (pEKE2-ptsH), when the extract was treated with alkaline phosphatase before the SDS-PAGE (Fig. S4). This observation indicates that the band with a molecular mass of 14 kDa represents a phosphorylated species of HPr. Independent of the growth phase for extracts of C. glutamicum ∆ptsH (pEKE2-ptsH-H15A) only one single band with a molecular mass of 14 kDa was detected (Fig. 2c). This finding indicates that HPr in C. glutamicum is exclusively phosphorylated at the His-15 residue required for the phosphorylation. Taken together, these results show that the functional PTS phosphorylation by EI and HPr involving phosphorylation of HPr at His-15 is required for optimal growth of C. glutamicum on maltose.

Maltose uptake is not inhibited in PTS-deficient C. glutamicum strains

In both EI- and HPr-deficient E. coli strains maltose uptake by the MalEFGK2 transporter is inhibited, because the unphosphorylated EIIClGlc binds to the MalK ATPase subunits and, thus, prevents structural rearrangements necessary for ATP hydrolysis and maltose transport (Bao & Duong, 2013; Chen et al., 2013). This inhibition of maltose uptake by unphosphorylated EIIClGlc also takes place when glucose is taken up via the PTS and is the underlying mechanism of inducer exclusion. The inhibition of maltose transport by EIIClGlc in EI- and HPr-deficient E. coli strains can be overcome by additional deletion of crr, which encodes EIIClGlc (Saier & Roseman, 1976a, b). In complementation studies, introduction of a gene fragment for the expression of the EIIA domain of the ptsG encoded, fused EIIClBClGlc permease from C. glutamicum restored growth on glucose of a crr-deficient E. coli strain (Lee et al., 1994). However, additional deletion of ptsG in C. glutamicum ∆ptsH did not lead to improved growth of the resulting strain C. glutamicum ∆ptsH ∆ptsG on maltose when compared to the parental strain (Fig. S5). In transport assays with 14C-labelled maltose, maltose uptake was slightly increased in C. glutamicum ∆ptsH when compared to WT (Fig. 3d). The increased uptake rates observed for C. glutamicum ∆ptsH might be attributed to the slowed-down metabolism. Upon ectopic expression of ptsH in C. glutamicum ∆ptsH (pEKE2-ptsH), the maltose uptake rate was reduced to the levels observed for C. glutamicum WT. Increased maltose uptake rates were also observed for the empty vector control C. glutamicum ∆ptsH (pEKE2) (Fig. 3d). For C. glutamicum ∆ptsI a maltose uptake rate of 19.3 ± 1.4 nmol min⁻¹ (mg cell dry weight)⁻¹ was determined, which is also slightly increased when compared to the maltose uptake rate determined for C. glutamicum WT (15.5 ± 1.3 nmol min⁻¹ (mg cell dry weight)⁻¹). These results show that the growth defect of PTS-deficient C. glutamicum strains in cultivations on maltose is probably not caused by the inhibition of maltose uptake, which would have been indicative for the presence of inducer-exclusion-like mechanisms in C. glutamicum.

MaIP activity, maIP RNA levels and expression of a maIP-gfp transcriptional fusion are reduced in C. glutamicum ∆ptsH

As the limitation of maltose uptake was excluded as the underlying reason for the poor growth of PTS-deficient C. glutamicum strains on the non-PTS sugar maltose, the activities of enzymes described to be involved in maltose utilization, namely MalQ, Glk, Pgm, pyruvate kinase (Pyk; Netzer et al., 2004) and MaIP, were investigated in
Fig. 3. (a) Growth of *C. glutamicum* WT (●) and ΔptsHr (○) in minimal medium with 1 % (w/v) maltose. (b) Growth of *C. glutamicum* ΔptsH (pEKEx2) (▼), ΔptsH (pEKEx2-ptsH) (△) and ΔptsH (pEKEx2-ptsH-H15A) (■) in minimal medium with maltose plus 250 μM IPTG. Three independent cultivations were performed; data from one representative experiment are shown, the results of each of the cultivations were comparable. (c) Western blot analysis with antibodies against the FLAG-epitope present in the HPr variants encoded by pEKEx2-ptsH and pEKEx2-ptsH-H15A with cell-free extract from *C. glutamicum* ΔptsH (pEKEx2-ptsH) and ΔptsH (pEKEx2-ptsH-H15A) cells cultivated on maltose. Cells were harvested after 4 and 24 h of cultivation. (d) Rates of [14C]-maltose uptake of *C. glutamicum* WT, ΔptsH, ΔptsH (pEKEx2) and ΔptsH (pEKEx2-ptsH) cultivated in minimal medium with 2 % (w/v) maltose; data represent mean values and sds of three independent measurements from three independent cultivations. Plasmids were induced by the addition of 250 μM IPTG.
cell-free extracts of *C. glutamicum* strains. In contrast to the activities of MalQ, Glk, Pgm and Pyk, MalP activity was significantly reduced in extracts of *C. glutamicum ΔptsH* when compared to WT (Table 2). Full MalP activity was restored upon ectopic expression of *ptsH* in the strain *C. glutamicum ΔptsH* (pEKEx2-ptsH), whereas MalP activity remained low in the empty vector control *C. glutamicum ΔptsH* (pEKEx2). In addition, transcript levels for *malp* and *malQ*, and for *musK*, *musE* and *musFGI*, which encode the components of the maltose transporter (Henrich *et al.*, 2013), were analysed in slot blot experiments with gene-specific RNA probes. Whereas just weak signals for *malp* were detected in RNA preparations from *C. glutamicum ΔptsH* and *ΔptsH* (pEKEx2), strong *malp* signals were detected for preparations from the strains *C. glutamicum* WT and *ΔptsH* (pEKEx2-ptsH) (Fig. 4a), which both possess functional PTS. For the genes *malQ*, *musK*, *musE* and *musG*, no apparent differences in signal intensities were detected in preparations from *C. glutamicum* WT, *ΔptsH*, *ΔptsH* (pEKEx2) and *ΔptsH* (pEKEx2-ptsH). To further analyse transcriptional regulation of the *malp* gene, a transcriptional fusion of the *malp* promoter and the promoterless *gfp* gene was constructed in the promoter probe vector pEPRI. The resulting plasmid pEPRI-PRmalP was introduced into *C. glutamicum* WT and *ΔptsH* and the fluorescence of GFP was analysed in the course of cultivation of the derived strains in minimal medium with maltose as the sole carbon substrate. Whereas the relative fluorescence intensities detected for *C. glutamicum* (pEPRI-PRmalP) increased in the course of cultivation, the relative fluorescence intensities detected for *C. glutamicum ΔptsH* (pEPRI-PRmalP) remained constantly low (Fig. 4b). Fluorescence intensities for both strains were comparably low after cultivation in TY complex medium (data not shown), as transcription of *malp* is not induced in cultivations of *C. glutamicum* on complex media such as TY (Clermont *et al.*, 2015), which contains no maltose. In conclusion, these data indicate that *malp* transcription and, therefore, also activity of the central enzyme for maltose utilization MalP depends on a functional PTS in *C. glutamicum*.

### Transcription of *malp* is derepressed in a suppressor mutant of HPr-deficient *C. glutamicum* growing fast with maltose

*C. glutamicum ΔptsH* was propagated in minimal medium with 1 % maltose as the sole source of carbon to generate suppressor mutants. After 10 consecutive cultivations in minimal medium with maltose aliquots of the culture were plated on LB agar plates and incubated at 30 °C for 16 h. To obtain a pure culture of the putative suppressor mutant strain, one of the colonies formed on the LB plates was further propagated by plating on LB agar plates for two more times. The obtained isolate, named *C. glutamicum* HSM, was then tested for growth with maltose or sucrose. *C. glutamicum* HSM grew in minimal medium with maltose as a substrate with a growth rate of 0.32 ± 0.04 h⁻¹ (Fig. 5a). This is about three times the rate of the parental strain *C. glutamicum ΔptsH* and nearly the same growth rate as the rate observed for *C. glutamicum* WT.

As expected for *ptsH*-deficient *C. glutamicum* strains in control experiments, *C. glutamicum* HSM was not able to grow in minimal medium with sucrose as the sole carbon source (Fig. 5b). Furthermore, deletion of *ptsH* in *C. glutamicum* HSM was confirmed using PCR with the primer pair Δhpr-Ver-fw and Δhpr-Ver-rv. As expected in samples from reactions with DNA from *C. glutamicum* HSM, as well as *C. glutamicum ΔptsH*, single bands of about 600 bp were detected, whereas a single band of about 800 bp was detected for PCRs with DNA from *C. glutamicum WT* as the template. Taken together, these results show that *C. glutamicum* HSM is indeed a PTS-deficient suppressor mutant strain able to grow well in minimal medium with maltose as the sole carbon substrate. MalP activity in extracts of *C. glutamicum* HSM was found to be restored to the level observed for *C. glutamicum* WT [a MalP activity of 0.14 ± 0.02 U (mg protein)⁻¹ was determined for *C. glutamicum* HSM], which is more than 2.5-fold higher than the activity of 0.06 ± 0.02 U (mg protein)⁻¹ determined for the parental strain *C. glutamicum ΔptsH* (Table 2). Growth of several *C. glutamicum* suppressor mutants could be explained by the occurrence of promoter-up mutations for genes limiting growth of the parental strains (Lindner

### Table 2. Specific activities [U (mg protein)⁻¹] of glucokinase (Glk), amyloglucosidase (MalQ), phosphoglucomutase (Pgm) pyruvate kinase (Pyk) and maltodextrin phosphorylase (MalP) in cell-free extracts of *C. glutamicum* WT, *ΔptsH*, *ΔptsH* (pEKEx2) and *ΔptsH* (pEKEx2-ptsH) cells cultivated in minimal medium containing 1 % maltose (w/v) as the carbon source

Cells were pre-cultivated in LB medium, inoculated with an OD₆₀₀ of 1.5 in minimal medium with maltose as the sole carbon source and harvested for analyses after reaching an OD₆₀₀ of 6. Data represent mean values and sds from three independent cultivations with three technical replicates.

<table>
<thead>
<tr>
<th>Activity</th>
<th>WT</th>
<th>ΔptsH</th>
<th>ΔptsH (pEKEx2)</th>
<th>ΔptsH (pEKEx2-ptsH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glk</td>
<td>0.05 ± 0.03</td>
<td>0.06 ± 0.03</td>
<td>0.05 ± 0.03</td>
<td>0.05 ± 0.03</td>
</tr>
<tr>
<td>MalQ</td>
<td>0.16 ± 0.04</td>
<td>0.17 ± 0.06</td>
<td>0.17 ± 0.04</td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td>Pgm</td>
<td>0.23 ± 0.05</td>
<td>0.32 ± 0.07</td>
<td>0.31 ± 0.08</td>
<td>0.23 ± 0.09</td>
</tr>
<tr>
<td>Pyk</td>
<td>0.03 ± 0.01</td>
<td>0.02 ± 0.01</td>
<td>0.02 ± 0.01</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>MalP</td>
<td>0.15 ± 0.05</td>
<td>0.06 ± 0.02</td>
<td>0.05 ± 0.02</td>
<td>0.14 ± 0.06</td>
</tr>
</tbody>
</table>
No mutations were observed in sequence analyses of the malP promoter region, as well as of the malP gene itself, in DNA preparations from C. glutamicum HSM; thus, no evidence for cis-acting mutations was found. To test for trans-acting mutations and to analyse whether malP transcription is changed in C. glutamicum HSM, the malP promoter test plasmid pEPRI-PR malP was used to transform C. glutamicum HSM. In cultivations on maltose, increased GFP fluorescence was determined for C. glutamicum HSM (pEPRI-PR malP) when compared to C. glutamicum D ptsH (pEPRI-RP malP), showing the increased transcription of the malP promoter in the suppressor mutant (Fig. 5c). Despite increased malP transcription, growth of C. glutamicum HSM (pEPRI-PR malP) in minimal medium with maltose proceeded significantly slower than growth of C. glutamicum HSM, as well as of the empty vector control strain C. glutamicum ΔΔptsH, ΔptsH (pEKEx2) and ΔptsH (pEKEx2-ptsH) cultivations in minimal medium with 1 % (w/v) maltose as the sole carbon source. RNA hybridization experiments were performed with specific DIG-labelled antisense RNA probes. Cells for RNA preparation were harvested after reaching an OD₆₀₀ of 6, one representative experiment of a series of three individual experiments is shown. (b) Relative fluorescence of the GFP reporter upon expression of the gfp gene under the control of the malP promoter measured in WT (black bars) and ΔptsH (grey bars) strains in the course of cultivation in minimal medium with 1 % (w/v) maltose. Data represent mean values and SDs of three independent measurements each from three independent cultivations.

Fig. 4. (a) Analysis of musFGI, musE, musK, malQ and malP transcript levels in RNA samples from C. glutamicum WT, ΔptsH, ΔptsH (pEKEex2) and ΔptsH (pEKEex2-ptsH) cultivations in minimal medium with 1 % (w/v) maltose as the sole carbon source. RNA hybridization experiments were performed with specific DIG-labelled antisense RNA probes. Cells for RNA preparation were harvested after reaching an OD₆₀₀ of 6, one representative experiment of a series of three individual experiments is shown. (b) Relative fluorescence of the GFP reporter upon expression of the gfp gene under the control of the malP promoter measured in WT (black bars) and ΔptsH (grey bars) strains in the course of cultivation in minimal medium with 1 % (w/v) maltose. Data represent mean values and SDs of three independent measurements each from three independent cultivations.
activator for the transcription of malP exists in C. glutamicum, which is controlled by the PTS.

**DISCUSSION**

In addition to its primary function for sugar uptake and phosphorylation, the PTS participates in various bacteria in the control of diverse cellular functions, such as catabolite repression, chemotaxis and control of pathogenesis (Deutscher et al., 2006, 2014; Gabor et al., 2011; Görke & Stülke, 2008). This regulatory role of the PTS seems obvious as the phosphorylation state of PTS components can be indicative for both the metabolic state of the organism and the types of carbon sources available. Despite its well-investigated role in substrate uptake in C. glutamicum (Moon et al., 2005; Parche et al., 2001;

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**Fig. 5.** (a, b) Growth of C. glutamicum WT (●), ΔptsH (○) and the HPr-deficient suppressor mutant HSM (▼) in minimal medium with 1 % (w/v) maltose (a) or 1 % (w/v) sucrose (b). Three independent cultivations were performed; data from one representative experiment are shown, results of each of the cultivations were comparable. (c) Relative fluorescence of the GFP reporter upon expression of the gfp gene under the control of the malP promoter measured in C. glutamicum ΔptsH (grey bars) and the HPr-deficient suppressor mutant strain C. glutamicum HSM (black bars) in the course of cultivation in minimal medium with 1 % (w/v) maltose. Data represent mean values and SDs of three independent measurements each from three independent cultivations.
Uhde et al., 2013), hitherto no regulatory functions of the PTS were described in this organism. Slowed-down growth of PTS-deficient C. glutamicum strains on maltose already had been observed by Parche et al. (2001). As these mutant strains were generated by incubation with toxic glucose analogues and, furthermore, the uptake system for maltose was still unknown in C. glutamicum, random inactivation of the gene for the maltose transporter and/or participation of the PTS in maltose uptake could not be excluded as the underlying reason for the observed slower growth. Based on the recent identification of the musEFGK1-encoded ABC transporter as the sole uptake system for maltose in C. glutamicum (Henrich et al., 2013), maltose uptake by the PTS could be excluded and, therefore, a regulatory function of the PTS for the control of maltose metabolism assumed. In contrast to the situation in E. coli, in which the activity of the ABC transporter for maltose is controlled by the PTS (Bao & Duong, 2013; Blüscbke et al., 2006; Böhm et al., 2002; Chen et al., 2013; Saier & Roseman, 1976a, b), maltose uptake was not negatively influenced in PTS-deficient C. glutamicum strains. However, we found in C. glutamicum that transcription of malP, which encodes an α-glucan phosphorylase required for maltose utilization (Seibold et al., 2009), depends on PTS activity. Besides maltose utilization, the malP-encoded α-glucan phosphorylase contributes in C. glutamicum to glycogen degradation. In cultivations with sugars, C. glutamicum transiently accumulates large amounts of glycogen in the early exponential growth phases, which are degraded in the course of the late exponential growth phase just before the initially provided substrate is consumed (Seibold et al., 2007; Seibold & Eikmanns, 2007). Besides slowed down growth on maltose, the deletion of malP in C. glutamicum causes a delayed degradation of glycogen in cultivations on glucose (Seibold et al., 2009). The recently identified control of C. glutamicum MalP by competitive inhibition by the glycogen synthesis intermediate ADP-glucose (a typical feature of glycogen phosphorylases) also pointed out a prominent role of this enzyme in the control of the glycogen content (Clermont et al., 2015). MalP has been shown to be also subject to transcriptional regulation, which responds to the substrate used for cultivation as well as the growth phase (Clermont et al., 2015). In cultivations on substrates requiring gluconeogenesis (e.g. acetate), MalP activity and malP transcription are low, whereas transiently high amounts of enzyme activity and transcript levels are found in C. glutamicum cells cultivated on sugars such as glucose and maltose. In detail, malP transcript levels and MalP activity increase in the course of the exponential growth phase (Clermont et al., 2015), and both parameters reach their maxima when the glycogen accumulated in the early exponential growth phase becomes degraded before the onset of the stationary growth phase (Seibold et al., 2007). At the onset of the stationary phase, when the initially provided glycolytic substrate has been consumed and the transiently accumulated glycogen has been degraded, malP transcript levels and MalP activity decrease (Clermont et al., 2015). In contrast to the situation in E. coli, in which malP transcription is activated via the transcriptional activator MalT by glycogen degradation products in the course of cultivation with glucose (Dippel et al., 2005; Ehrmann & Boos, 1987), activation of malP transcription occurs in C. glutamicum in cultivations on glycolytic substrates independent of the glycogen formation (Clermont et al., 2015). Based on the here observed interconnection between a functional PTS and control of malP transcription and the previously observed carbon-source-dependent regulation of malP transcription, it is tempting to speculate that the phosphorylation status of PTS components might act as a stimulus for the activation of malP transcription. By this means, the enzymic setup of the cell might be adjusted to the presence of a glycolytic carbon source, whereas the actual control of the glycogen degradation is brought about by the recently described direct inhibition of MalP by ADP-glucose (Clermont et al., 2015). However, this hypothetical scheme for the control of the glycogen metabolism in C. glutamicum remains to be verified. In E. coli the PTS directly controls activity of the α-glucan phosphorylase GlgP, as dephosphorylated HPr binds and activates GlgP (Seok et al., 1997). It seems a reasonable strategy for bacteria to use the PTS as a sensor for the coordination of substrate availability and glycogen metabolism. Taken together we here show that the PTS of C. glutamicum, in addition to its function as a transporter, contributes to the control and coordination of carbohydrate metabolism in this organism.

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