The Gram-positive *Corynebacterium glutamicum* efficiently metabolizes maltose by a pathway involving maltodextrin and glucose formation by 4-α-glucanotransferase, glucose phosphorylation by glucose kinases, and maltodextrin degradation via maltodextrin phosphorylase and α-phosphoglucomutase. However, maltose uptake in *C. glutamicum* has not been investigated. Interestingly, the presence of maltose in the medium causes increased expression of *ptsG* in *C. glutamicum* by an unknown mechanism, although the *ptsG*-encoded glucose-specific EII permease of the phosphotransferase system itself is not required for maltose utilization. We identified the maltose uptake system as an ABC transporter encoded by *musK* (cg2708; ATPase subunit), *musE* (cg2705; substrate binding protein), *musF* (cg2704; permease), and *musG* (cg2703; permease) by combination of data obtained from characterization of maltose uptake and reanalyses of transcriptome data. Deletion of the *mus* gene cluster in *C. glutamicum* abolished maltose uptake and utilization. Northern blotting and reverse transcription-PCR experiments revealed that *musK* and *musE* are transcribed monocistronically, whereas *musF* and *musG* are part of an operon together with *cg2701* (*musI*), which encodes a membrane protein of unknown function with no homologies to characterized proteins. Characterization of growth and [14C]maltose uptake in the *musI* insertion strain *C. glutamicum IM*cg2701 showed that *musI* encodes a novel essential component of the maltose ABC transporter of *C. glutamicum*. Finally, *ptsG* expression during cultivation on different carbon sources was analyzed in the maltose uptake-deficient strain *C. glutamicum IM*. Indeed, maltose uptake by the novel ABC transport system MusEFGK2I is required for the positive effect of maltose on *ptsG* expression in *C. glutamicum*.
analyzed the substrate-dependent influence of the maltose uptake system on ptsG expression.

MATERIALS AND METHODS

Microorganisms, plasmids, oligonucleotides, and cultivation conditions. C. glutamicum and E. coli strains and their relevant characteristics are listed in Table 1. Plasmids, their relevant characteristics and sources, and oligonucleotides used in this study are listed in Table 2 and Table S1 in the supplemental material. The CgC minimal medium used for the cultivation of C. glutamicum has been described previously (30) and contained maltose, maltotriose, glucose, and/or acetate at concentrations indicated in Results. TY broth (31) was utilized as complex medium for cultivation experiments. The C. glutamicum strains and their relevant characteristics are listed in Table 1.

TABLE 1 Strains used in this study

<table>
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<th>Strain</th>
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<td>E. coli</td>
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<td>Δmus</td>
<td>In-frame deletion of genes cg2708 to cg2703 of C. glutamicum WT</td>
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<td>IMcg2708</td>
<td>C. glutamicum WT with insertion of pDrive in cg2708</td>
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<td>ΔptsG</td>
<td>In-frame deletion of ptsG gene (cg1537) of C. glutamicum WT</td>
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TABLE 2 Plasmids used in this study

<table>
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<td>pK19mobSacB</td>
<td>Kanr, mobilizable E. coli vector for the construction of insertion and deletion mutants in C. glutamicum (oriV, sacB, lacZa)</td>
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<td>pET2-PrptsG</td>
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Previously (34). In detail, the flanking regions of the gene/cluster were amplified using primer pairs Δ gene/cluster_1 plus Δ gene/cluster_2 and Δ gene/cluster_3 plus Δ gene/cluster_4 (see Table S1 in the supplement material). The two flanking PCR products obtained served as the templates for a crossover PCR using the primer pair Δ gene/cluster_1 plus Δ gene/cluster_4. The resulting PCR product was digested with the enzymes indicated in Table S1 and cloned into pK19mobSacB cut with the same enzymes. Gene deletion with the derived pK19mobSacB Δ gene/cluster plasmids was carried out as described previously (34). The deletion of the cluster comprising open reading frames (ORFs) cg2708 to cg2703 was verified by PCR using the primer pair check MusREV and check MusFOR, resulting in a 7,018-bp fragment for the wild type (WT) and a 1,188-bp fragment for the mus deletion mutant; pstG deletion was verified using the primers check ΔpstGFOR and check ΔpstGREV, resulting in a 3,583-bp fragment for the WT and a 1,867-bp fragment for the deletion mutant.

Insertion mutagenesis was applied for the generation of single-gene mutants, using vector pDrive as recently described (28). For this purpose, internal fragments of the loci were amplified by PCR using the primers IM-orf-fw and IM-orf-rev (see Table S1 in the supplement material) and cloned into vector pDrive according to the manufacturer’s instructions. The resulting plasmids were isolated and used for gene disruption as described previously (35). Integration into the genome in the resulting strains was verified by PCR using gene-specific primers CONTR-orf (see Table S1) and M13-FP.

Construction of expression vectors. For isopyrrol-β-β-thiogalactopyranoside (IPTG)-inducible overexpression, vector pXMJ19 was used (36). Genes were amplified via PCR from genomic DNA of C. glutamicum ATCC 13032 (37) using the oligonucleotide primers listed in Table S1 in the supplement material. 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, and the resulting fragments were ligated into the plasmid pXMJ19 (digested with the same enzymes) and transformed into E. coli DH5α. The resulting plasmids were isolated and the nucleotide sequences controlled by sequencing (GATC Biotech).

Cloning of the musF promoter. The promoter probe vector pET2 was used to construct a transcriptional fusion of the musF (cg2704) promoter to the promoterless cat gene. The musF promoter fragment was amplified by PCR with the primers PRmusF-for and PRmusF-rev. The 380-bp PCR product, covering the region from 226 bp upstream to 133 bp downstream of the musF translational start codon, was digested with XbaI and BamHI and ligated into the multiple cloning site (MCS) in front of the cat gene in pET2, resulting in pET2-PRmusF. Furthermore, plasmid pET2-Pmus-TS, which lacks the musF transcriptional start site, was cloned. Therefore, a shortened musF promoter fragment was generated by PCR using the primers PRmusF-TS-for and PRmusF-rev, and the 214-bp PCR product was digested with XbaI and BamHI and ligated into pET2.

Enzyme assays and protein analysis. To determine chloramphenicol acetyltransferase (CAT) activity, C. glutamicum cells were harvested, washed twice in 0.1 M Tris-HCl, pH 7.8, and resuspended in the same buffer containing 10 mM MgCl2 and 1 mM EDTA. The specific CAT activity was determined as described by Schreiner et al. (38). Protein concentrations were determined using the Rmri-Nanokoukit (Roth) with bovine serum albumin as the standard. SDS-PAGE was performed according to Laemmli (39). Loading buffer (4×) contained 8% (wt/vol) SDS, 20% (vol/vol) glycerol, 10 mM EDTA, 100 mM Tris-HCl, pH 6.8, 2% (vol/vol) β-mercaptoethanol, and 1 mg/mL bromphenol blue. Membrane preparations and Western blot experiments for detection of the Streptavidin-tagged Cg2701 protein by using antibodies raised against the Streptag II (IBA GmbH) were performed as described for the uptake carrier BetP (40).

Protein purification and EMSAs. RamA was synthesized as hexahistidyl-tagged fusion proteins and purified by Ni2+ affinity chromatography as described previously (41). The binding of purified RamA protein was tested by electrophoretic mobility shift assays (EMSAs) using DNA fragments generated by PCR and purified using the Nucleospin extract kit (Macherey-Nagel). The 380-bp fragment musF-Pr, carrying the musE-musF intergenic region, was amplified using primer pair PRmusF-for and PRmusF-rev. The 211-bp fragment ramBp3b, generated with the primer pair ramBp3b_forw and ramBp3b_rev, was used as a negative control for RamA binding (42). In the binding assays, 10 to 300 ng of the fragments was incubated with various amounts of RamA (0 to 7.5 μg) in 20 μl 10 mM Tris-HCl reaction buffer, pH 7.6, containing 50 mM NaCl, 1 mM dithiothreitol (DTT), 1 mM EDTA, 10% (wt/vol) glycerol, and 1 μg poly(d[I-C]) for 20 min at room temperature. Subsequently, the mixture was separated on a 2% agarose gel in 1 TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA) at 70 V and 80 mA and stained with ethidium bromide.

RNA techniques. Isolation of total RNA from C. glutamicum cells was performed using the NucleoSpin RNAII kit (Macherey& Nagel) as described by Wolf et al. (43). For Northern (RNA) hybridization, digoxigenin (DIG)-11-dUTP-labeled gene-specific antisense RNA probes were prepared from PCR products (generated with oligonucleotides listed in Table S1 in the supplement material) carrying the T7 promoter by in vitro transcription (1 h, 37°C) using T7 RNA polymerase (MBI Fermentas). For hybridization, total RNA of C. glutamicum was separated on an agarose gel containing 17% (vol/vol) formaldehyde and transferred to a nylon membrane using the VacuGene system from Pharmacia. RNA was cross-linked to the membrane by means of UV irradiation at 125 J·cm−2. Hybridization and detection were carried out according to the DIG application manual (Roche Applied Science). Slot blot experiments were performed as described previously (44).

The transcriptional start site of cg2704 (musG) was determined using the 5′/3′-rapid amplification of cDNA ends (RACE) kit from Roche Diagnostics according to the manufacturer’s manual. First-strand cDNA was synthesized from 2 μg of total RNA using the gene-specific primer designated RACE-cg2704-SP1 in Table S1 in the supplement material. The subsequent PCRs were performed using the primer pair RACE-cg2704-SP2 oligonucleotide anchor primer (the latter is included in the kit). The purified PCR product was ligated into plasmid pDrive (Qiagen), resulting in the recombinant plasmid pDrive-RACE-cg2703, which was sequenced. The transcriptional site was deduced from the sequences obtained.

[14C]maltose uptake studies. Maltose uptake studies were performed essentially as described for glucose (28). In detail, C. glutamicum cells were grown 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 measurement. Before the transport assay, cells were incubated for 3 min at 30°C; the reaction was started by addition of 1 μM to 1 mM [14C]maltose (specific activity, 679 mCi μmol−1; Amersham, Braunschweig, Germany). Inhibitors were added at the concentrations indicated 30 s before the measurements were started. At given time intervals (15, 30, 45, 60, and 120 s), 200-μl samples were filtered through glass fiber filters (Type F; Millipore, Eschborn, Germany) and washed twice with 2.5 ml of 100 mM LiCl. Radioactivity of the samples was determined using scintillation fluid (Rotiszynth; Roth, Germany) and a scintillation counter (LS 6500; Beckmann, Krefeld, Germany). Kinetic parameters as well as standard errors were derived from nonlinear regressions according to the Michaelis-Menten equation by using Sigma Plot software.

Computational analysis. Databank searches were carried out by using BLAST (45) and the KEGG database (46). Identification of putative rhindependent transcriptional terminators was performed using TransTermHP (47), and MFold (48) was the software used for the calculation of their ΔG° values (change in Gibbs free energy under standard conditions). Topology predictions of membrane proteins were performed using both TMHMM (49) and SOSUI (50). Protein sequences were analyzed using CLUSTAL W (51).
RESULTS

Kinetic parameters and transport mechanism for maltose uptake. Uptake assays with C. glutamicum cells, cultivated in minimal medium with glucose as the sole carbon source and using various concentrations (0.5 to 200 \mu M) of \[^{14}\text{C}\] labeled maltose, were performed for the determination of kinetic parameters. Maltose uptake in glucose-cultivated cells showed a simple saturation kinetics, with a \( K_m \) of 1.0 ± 0.2 \mu M and a \( V_{max} \) of 22.6 ± 0.8 mmol min\(^{-1}\) mg\(^{-1}\) cell dry matter (cdm) (see Fig. S1 in the supplemental material). Uptake of maltose in cells of the Ehi\(^{AS}\)-deficient strain C. glutamicum \( \Delta ptsG \) cultivated on TY complex medium proceeded with a rate of 27.8 ± 1.8 mmol min\(^{-1}\) mg\(^{-1}\) cdm, which is nearly identical to that of cells of C. glutamicum WT (29.4 ± 2.0 mmol min\(^{-1}\) mg\(^{-1}\) cdm), when cells were cultivated in TY and measurements were performed at a maltose concentration of 100 \mu M. Based on these results, it can be ruled out that the high-affinity uptake of maltose in C. glutamicum requires the ptsG-encoded permease.

To specify the class of uptake system mediating maltose transport, we measured the effects of selective inhibitors on \[^{14}\text{C}\] maltose uptake at a substrate concentration of 100 \mu M maltose. Simultaneous addition of valinomycin (ionophore for \( K^+ \)) and nigericin (ionophore for \( H^+ \) and \( K^+ \)) to C. glutamicum cells abolishes the proton-motive force necessary to drive secondary active transporters (52, 53). However, maltose uptake was only reduced to 17.0 ± 5.0 mmol min\(^{-1}\) mg\(^{-1}\) cdm when both inhibitors were present simultaneously in the assay (not shown). Addition of vanadate, which inhibits ATP-dependent primary active transporters, to the assays inhibited maltose uptake in C. glutamicum (11.0 ± 5.0 mmol min\(^{-1}\) mg\(^{-1}\) cdm; experiments not shown). Therefore, it seems feasible that maltose uptake in C. glutamicum is mediated by a high-affinity ATP-binding cassette (ABC) transport system.

Factors affecting maltose uptake in C. glutamicum. To identify putative parameters affecting maltose uptake properties in C. glutamicum, \[^{14}\text{C}\] maltose uptake in cells cultivated in minimal medium with different carbon sources was analyzed. Rates for \[^{14}\text{C}\] maltose uptake were slightly lower in cells cultivated with maltose than in cells cultivated with glucose as the sole carbon source (17.0 ± 6.4 and 22.7 ± 3.4 mmol min\(^{-1}\) mg\(^{-1}\) cdm, respectively). Reduced \[^{3}\text{H}\] maltose uptake rates were observed for cells cultivated on lactate (13.3 ± 1.5 mmol min\(^{-1}\) mg\(^{-1}\) cdm), on fructose (10.7 ± 1.7 mmol min\(^{-1}\) mg\(^{-1}\) cdm), or on acetate (10.7 ± 1.0 mmol min\(^{-1}\) mg\(^{-1}\) cdm). These differences of the observed uptake rates might be due to carbon source-dependent transcriptional regulation of the genes encoding the maltose uptake system in C. glutamicum.

Three transcriptional regulators, RamA, RamB, and SugR, have been shown to be master regulators for the adjustment of the central metabolism of C. glutamicum in response to the utilization of the gluconeogenic substrate acetate (reviewed in reference 54). We therefore analyzed \[^{14}\text{C}\] maltose uptake and maltose utilization by the RamA-deficient strain C. glutamicum \( \Delta ramA \), the RamB-deficient strain C. glutamicum \( \Delta ramB \), and the SugR-deficient strain C. glutamicum \( \Delta sugR \). As both uptake of (9.1 ± 0.3 mmol min\(^{-1}\) mg\(^{-1}\) cdm) and growth on (growth rate of 0.27 ± 0.01 h\(^{-1}\)) maltose were significantly reduced in C. glutamicum \( \Delta ramA \) compared to C. glutamicum WT (uptake rate, 30.3 ± 2.4 mmol min\(^{-1}\) mg\(^{-1}\) cdm; growth rate, 0.37 ± 0.03 h\(^{-1}\)), we concluded that RamA probably acts as an activator of genes for maltose utilization/uptake. Maltose uptake (10.8 ± 0.4 mmol min\(^{-1}\) mg\(^{-1}\) cdm) and growth on maltose (0.27 ± 0.02 h\(^{-1}\)) were also found to be slowed in C. glutamicum \( \Delta sugR \). This indicates that SugR also acts as an activator of genes for maltose utilization, which is rather unexpected, as this Deor-type transcriptional regulator has been described as a global repressor of genes required for carbohydrate utilization in C. glutamicum (reviewed in reference 54). Growth of the RamB-deficient strain C. glutamicum \( \Delta ramB \) in minimal medium with maltose (0.34 ± 0.02 h\(^{-1}\)) as well as maltose uptake (18.9 ± 1.5 mmol min\(^{-1}\) mg\(^{-1}\) cdm) for cells cultivated with glucose were only slightly slower than those of the parental strain C. glutamicum WT. These data suggest that RamB also is involved in the transcriptional regulation of the genes encoding the maltose uptake system.

Analyses of transcriptome data lead to identification of the maltose uptake system. Several RNA microarray studies of the transcriptomes of C. glutamicum WT, C. glutamicum \( \Delta ramA \), C. glutamicum \( \Delta ramB \), and C. glutamicum \( \Delta sugR \), as well as a chromatin immunoprecipitation (ChIP)-to-chip analysis of potential SugR-binding sites, were recently published (27, 55, 56, 57). Based on the results from the characterization of maltose uptake described above, the data from the RNA microarrays were analyzed to identify candidate genes for the maltose uptake system. The following criteria were used for the analyses. Genes encoding the maltose uptake system of C. glutamicum should (i) encode membrane proteins, preferably components of ABC transporters, (ii) be expressed in cultivations with glucose and repressed in cultivations with acetate, (iii) be induced by RamA and therefore repressed in the ramA deletion mutant C. glutamicum \( \Delta ramA \) when cultivated on glucose, (iv) be repressed in C. glutamicum \( \Delta sugR \), and (v) be slightly affected in the ramB deletion mutant C. glutamicum \( \Delta ramB \). We reanalyzed the above-mentioned transcriptome data using our set of criteria and identified two clusters of genes, cg2181 to cg2184 and cg2703 to cg2708, which indeed encode putative ABC transporters and which were repressed in C. glutamicum WT during cultivation with acetate, and they were also repressed in C. glutamicum \( \Delta ramA \) when cultivated with glucose. As the cluster cg2181 to cg2184 is annotated as a peptide uptake system (25), we focused on the investigation of the cluster cg2703 to cg2708, annotated as a putative ABC-type sugar transporter. In detail, cg2703 and cg2704 probably encode the transmembrane domains (TMDs), cg2705 the substrate binding domain, cg2708 the nucleotide binding domains (NBDs), and cg2707 a hypothetical protein.

The cluster of genes from cg2703 to cg2708 was deleted, and the resulting mutant strain, C. glutamicum \( \Delta mus \), was tested for both growth with and uptake of maltose. As depicted in Fig. 1A and B, both utilization and uptake of maltose were abolished in C. glutamicum \( \Delta mus \). At least partial complementation of this phenotype was achieved by the introduction of plasmid pXMJ19-musKEFG, which carries the complete gene cluster from cg2703 to cg2708. Introduction of the empty vector pXMJ19 into C. glutamicum \( \Delta mus \) did not restore growth on or uptake of maltose (Fig. 1B). From these experiments, we conclude that the gene cluster comprising cg2703 to cg2708 indeed encodes the single, high-affinity maltose uptake system of C. glutamicum; therefore, we named the genes for the components of this ABC transporter musK (cg2708), musE (cg2705), musF (cg2704), and musG (cg2703).

Maltodextrin uptake by the \( \text{musKEFG} \)-encoded ABC transport system. To analyze the substrate specificity of the ABC trans-
porter encoded by the musKEFG genes, [14C]maltose uptake assays were performed with unlabeled competitors in 100-fold excess. As depicted in Fig. 2A, addition of 50 mM unlabeled maltose to the uptake assay completely quenched the uptake of label. No effects on [14C]maltose uptake were observed by addition of glucose or the disaccharides trehalose and isomaltose. Addition of the pseudo-oligosaccharide maltotetraose completely quenched the uptake of [14C]maltose; furthermore, maltopen-taose reduced the [14C]maltose uptake rate from 19.5 ± 1.5 nmol min⁻¹ mg⁻¹ cdm to 6.8 ± 0.3 nmol min⁻¹ mg⁻¹ cdm. Addition of maltodextrins consisting of more than five glucose moieties, such as maltohexaose and maltoheptaose, did not affect the uptake of labeled maltose. Addition of the pseudo-oligosaccharide acarbose, which in *E. coli* is taken up via the maltose ABC transporter and inhibits maltose metabolism (58), had no effect on the uptake of labeled maltose in *C. glutamicum*. Taken together, these data indicate that in addition to maltose, short maltodextrins probably are also substrates of the musKEFG-encoded ABC transport system.

However, the assay utilized here does not discriminate between competition for transport or only binding. To analyze if short maltodextrins are indeed substrates of the musKEFG-encoded ABC transport system, growth experiments with 0.5% (wt/vol) maltopentaose as the sole substrate were performed. As shown in Fig. 2B, *C. glutamicum* utilized maltotriose as a carbon source, grew at a rate of 0.38 ± 0.02 h⁻¹, and reached a final optical density of 14.0 ± 0.5 after 24 h of cultivation. Neither growth nor maltotriose utilization was observed for *C. glutamicum Δmus* (Fig. 2B). Maltotetraose was efficiently used as the sole source of carbon and energy by *C. glutamicum*; in growth experiments with 0.5% (wt/vol) maltotetraose, a growth rate of 0.34 ± 0.02 h⁻¹ and a final optical density of 10.5 ± 0.5 were measured. No growth on maltotetraose was observed for *C. glutamicum Δmus*. In growth experiments with 0.5% (wt/vol) malto-pentaose as the sole substrate, no growth of *C. glutamicum* was detected. From these results, we conclude that in addition to maltose, the short maltodextrins maltotriose and maltotetraose are taken up in *C. glutamicum* via the musKEFG-encoded ABC transport system.

Transcriptional organization of the musKEFG genes. At first glance, in *C. glutamicum* the genes encoding the maltose/maltodextrin uptake system seem to be organized as an operon (Fig. 3A), which may comprise the two additional open reading frames cg2707 and cg2701. The two open reading frames cg2707 and cg2701 are annotated as genes for hypothetical proteins (25). In accordance with this, initial analyses of the genome sequence revealed the presence of a rho-independent transcriptional terminator upstream (centered 143 bp upstream of the musK ATG start codon; ΔG°⁺, −21.9 kcal mol⁻¹; TT1) (Fig. 3A) and of a transcriptional terminator downstream of the mus genes (centered 1,058 bp downstream of the TAA stop codon of cg2701, the ORF directly following musG; ΔG°⁺, −13.6 kcal mol⁻¹; TT2). To further investigate the transcriptional organization, we employed Northern blot analyses with specific RNA probes raised against musK, cg2707, musE, musG, and cg2701. As shown in Fig. 3B, with the probes specific for musK and musE, respectively, signals were detected which correspond to fragment sizes of about 1,800 nucleotides. No signal was detected with the cg2707-specific probe (data not shown). These data show that musK and musE are transcribed monocistronically, and that there is no single transcript for all mus genes, as the whole cluster (musK to musG) comprises about 6,000 nucleotides (Fig. 3A). With the probes for musF and cg2701, transcripts of about 2,500 bases were detected (Fig. 3B). From these data it can be concluded that musF, musG, and cg2701 form an operon, as these genes comprise 2,427 bp. The organization of the musF-musG-cg2701 operon was confirmed by reverse transcription-PCR (RT-PCR) analyses, which clearly showed musF and musG as well as musG and cg2701 being coexpressed (data not shown). Furthermore, employing 5’-RACE with total RNA of maltose-grown *C. glutamicum* WT cells, a transcriptional start site of the musF-musG-cg2701 operon was determined (results not
results, uptake of $^{14}$C-labeled maltose was also abolished in both the sole carbon source. In conformity with the above-mentioned $\mu$sF maltodextrins were open to question. To investigate the individual $cg2701$ C. glutamicum named blot analysis of RNA samples from C. glutamicum, we carried out complementation studies with the plasmid pXMJ19-cg2701. As shown in Fig. 4A, ectopic expression of $cg2701$ in C. glutamicum IMcg2701 by the plasmid pXMJ19-cg2701 restored growth with maltose as the sole carbon source, while no growth was observed for the strain carrying the empty plasmid pXMJ19. In accordance with these results, uptake of $^{14}$C-labeled maltose was observed for C. glutamicum IMcg2701(pXMJ19-cg2701) (maltose uptake rate, 16.7 ± 2.3 nmol min$^{-1}$ mg$^{-1}$ cdw; results not shown), while no uptake of label was detected for C. glutamicum IMcg2701 (pXMJ19). In addition, the introduction of the plasmid pXM19-cg2701-strep for expression of cg2701 as a C-terminally tagged protein also restored growth with maltose as the sole carbon source (Fig. 4A) and uptake of maltose ($17.1 ± 1.9$ nmol min$^{-1}$ mg$^{-1}$ cdw) in C. glutamicum IMcg2701. The tagged protein Cg2701-Strep was detected in Western blotting experiments with antibodies binding to the N-terminal tag exclusively in the membrane fraction of C. glutamicum IMcg2701(pXM19-cg2701-strep) cells (Fig. 4B).

The finding that cg2701 is required for maltose uptake in C. glutamicum made us question the above-described results of the complementation studies with C. glutamicum $\mu$sK using plasmid pXMJ19-mus, as this plasmid only harbors the genes musK to $\mu$sG and did not lead to full complementation. However, the plasmid-encoded expression of all genes of the mus cluster ($musK$, $musE$, $musF$, and $musG$), together with cg2701 by plasmid pXMJ19-musEGFK1 in C. glutamicum $\mu$sK, did not further improve growth on maltose, as identical growth rates for C. glutamicum $\mu$sK(pXMJ19-musEGFK1) and C. glutamicum $\mu$sK(pXMJ19-musEGFKG) were observed ($0.26 ± 0.02$ h$^{-1}$ and $0.27 ± 0.03$ h$^{-1}$, respectively). These data seem to be contradictory to the above-described transcriptional organization of musF, musG, and cg2701 as an operon, since no transcript of cg2701 should be present in C. glutamicum $\mu$sK(pXMJ19-musEGFKG) cells. As cg2701 is essential for maltose uptake, the aforementioned strain should not grow on maltose. However, in RNA slot blot experiments using a cg2701-specific probe, transcripts of cg2701 were detected in both C. glutamicum $\mu$sK and C. glutamicum $\mu$sK(pXMJ19-musEGFKG) (data not shown). Indeed, stronger signals for cg2701 expression were detected for the C. glutamicum WT and for C. glutamicum $\mu$sK(pXMJ19-musEGFKG), which carries the plasmid comprising the complete mus gene clus-

![FIG 3 Genetic map of the cg2708 to cg2701 locus in C. glutamicum (A), Northern blot analysis of RNA samples from C. glutamicum cells grown in CgC minimal medium containing maltose as a carbon source (B), and growth of C. glutamicum WT (filled circles), C. glutamicum IMcg2708 (filled squares), C. glutamicum IMcg2707 (open squares), C. glutamicum IMcg2705 (filled triangles), C. glutamicum IMc2703 (open circles), and C. glutamicum IMcg2701 (open squares) in CgC minimal medium with 2% (wt/vol) maltose (C). Panel A shows the arrangements of the genes as arrows and the predicted transcriptional terminators (TT1 and TT2). For the Northern blot analyses, RNA was electrophoresed and probed with RNA probe specific for cg2708 (musK), cg2705 (musE), cg2703 (musG), or cg2701 (musI); the size of RNA fragments, determined with the RiboRuler high-range RNA ladder (Fermentas), is shown on the left. One representative growth curve of at least three independent cultivations is shown in panel C; the results of each of the cultivations were comparable.](http://jb.asm.org/)

shown). In three independent experiments, the transcriptional start site of musF (T$_S$$_\mu$sF) was found to be a cytosine residue within the musE-musF intergenic region, located 64 bp upstream of the musF ATG start codon and 122 bp downstream of the $\mu$sE TAA stop codon (see Fig. S2 in the supplemental material). Upstream of T$_S$$_\mu$sF we found a −10 CATCCT motif that only slightly matches the −10 consensus motif (TANANT) determined recently for corynebacteria (59); however, it is similar to the −10 regions of aceE (TATCCT [38]) and metE (CGTCTC [60]).

As the data presented here showed that the mus genes are not organized as a single operon, the functions of these genes as components of the C. glutamicum ABC transporter for maltose and maltodextrins were open to question. To investigate the individual functions of these genes, we constructed the single-gene disruption mutants of musK, cg2707, musE, musF, and cg2701, named C. glutamicum IMmusK, C. glutamicum IMcg2707, C. glutamicum IMmusE, C. glutamicum IMmusF, and C. glutamicum IMcg2701, respectively. As depicted in Fig. 3C, inactivation of both musF and musE resulted in the loss of growth with maltose as the sole carbon source. In conformity with the above-mentioned results, uptake of $^{14}$C-labeled maltose was also abolished in both C. glutamicum IMmusE and C. glutamicum IMmusF (results not shown). As both utilization of maltose as the sole carbon source (Fig. 3C) and uptake of $[^{14}$C]maltose were slowed down at least 5-fold in C. glutamicum IMmusK ($2.5 ± 0.5$ nmol min$^{-1}$ mg$^{-1}$ cdw; growth rate, $0.07 ± 0.5$ h$^{-1}$) compared to the parental strain C. glutamicum WT ($22.7 ± 3.4$ nmol min$^{-1}$ mg$^{-1}$ cdw; growth rate, $0.37 ± 0.01$ h$^{-1}$), we conclude that an alternative, unidentifiable ATPase can partially replace MusK. Inactivation of the ORF cg2707, which encodes a hypothetical soluble protein, did not affect maltose utilization (growth rate, $0.36 ± 0.02$ h$^{-1}$) (Fig. 3C) and uptake of maltose (maltose uptake rate for C. glutamicum IMcg2707 of $27.5 ± 2.8$ nmol min$^{-1}$ mg$^{-1}$ cdw). However, inactivation of the second putative gene, cg2701, severely impaired maltose metabolism of C. glutamicum, as growth on maltose (Fig. 3C) and maltose uptake were abolished in C. glutamicum IMcg2701. Taken together, these results show that although the genes musK, musE, musF, and musG are not part of a single operon, their gene products encode components of the ABC transporter for maltose uptake in C. glutamicum. The cg2701-encoded membrane protein MusI is an essential, novel component of the maltose ABC transporter of C. glutamicum. To rule out the possibility that the effect on maltose utilization in C. glutamicum IMcg2701 is due to polar effects on musG, we carried out complementation studies with the plasmid pXMJ19-cg2701. As shown in Fig. 4A, ectopic expression of cg2701 in C. glutamicum IMcg2701 by the plasmid pXMJ19-cg2701 restored growth with maltose as the sole carbon source, while no growth was observed for the strain carrying the empty plasmid pXMJ19. In accordance with these results, uptake of $^{14}$C-labeled maltose was observed for C. glutamicum IMcg2701(pXMJ19-cg2701) (maltose uptake rate, $16.7 ± 2.3$ nmol min$^{-1}$ mg$^{-1}$ cdw; results not shown), while no uptake of label was detected for C. glutamicum IMcg2701 (pXMJ19). In addition, the introduction of the plasmid pXM19-cg2701-strep for expression of cg2701 as a C-terminally tagged protein also restored growth with maltose as the sole carbon source (Fig. 4A) and uptake of maltose ($17.1 ± 1.9$ nmol min$^{-1}$ mg$^{-1}$ cdw) in C. glutamicum IMcg2701. The tagged protein Cg2701-Strep was detected in Western blotting experiments with antibodies binding to the N-terminal tag exclusively in the membrane fraction of C. glutamicum IMcg2701(pXM19-cg2701-strep) cells (Fig. 4B).
Taking these findings together, it can be concluded that cg2701 indeed encodes an essential component of the maltose/maltodextrin uptake system of C. glutamicum; therefore, we named the gene musI. Analyses of the MusI amino acid sequence and the predicted protein topology using both SOSUI and TMHMM revealed that MusI probably possesses an extracellular N terminus and 5 transmembrane helices (Fig. 4C); however, the cytoplasmic and periplasmic regions of MusI share no similarities with characterized proteins to date. Analyses of the deduced amino acid sequences and predicted protein topologies of the gene products of musF and musG showed that each of the two membrane proteins possesses six transmembrane domains and contains the so-called EAA sequence motifs required for interaction with the NBDs in the last cytoplasmic loop, as is common for TMDs of prokaryotic ABC importers (17, 61, 62). As TMDs of prokaryotic ABC importers contain up to 11 transmembrane helices (63), we assume that the additional protein MusI provides the additional transmembrane segments for one or both TMDs of the C. glutamicum maltose uptake system.

Effects of the transcriptional regulators RamA and SugR on musK, musE, and musFGI transcription. Characterization of growth and maltose uptake properties of the RamA-deficient strain C. glutamicum ΔramA and the SugR-deficient strain C. glutamicum ΔsugR pointed toward a strong role of the two regulators in the transcriptional control of the mus genes, as both maltose utilization and uptake were severely impaired in both mutant strains. The transcriptome data of the ramA and the sugR deletion mutants, reanalyzed here for the identification of the maltose uptake system, were derived from growth experiments on complex medium and/or minimal medium with glucose or acetate as the carbon source (55, 57). Hence, these transcriptome analyses provide no insights on transcriptional regulation of the mus genes toward the utilization of maltose as the sole carbon source. Therefore, we performed slot blot experiments with RNA probes raised against musK, musE, and musF and RNA samples derived from cultivations of C. glutamicum WT, C. glutamicum ΔramA, and C. glutamicum ΔsugR on glucose, maltose, or acetate. As shown in Fig. 5A, compared to the signals obtained for cultivations of C. glutamicum WT on maltose or glucose, only minor amounts of musE and musF transcripts were detected in C. glutamicum WT cells cultivated on acetate. No obvious changes in the amounts of detected musK transcripts were observed in the slot blot experiments with RNA samples from cultivations on different carbon sources (Fig. 5A). Analyses of the expression of musK, musE, and musF in the RamA-deficient strain C. glutamicum ΔramA showed that fewer transcripts of musE and musF were present in the mutat strain than in C. glutamicum WT; however, no altered expression was observed for musK. This effect of the lack of the transcriptional activator RamA on the expression of musE and musF was even more apparent in C. glutamicum ΔramA cells from cultivations with maltose than in cells from cultivations on glucose. These results correspond to the finding of putative RamA binding sites upstream of musE (ACCCCG; 19 bp upstream of the annotated TTG start codon) and of musF (CGGGGA and AGGGGA, 69 and 64 bp upstream of T_Sugar, respectively; the musF promoter region is depicted in Fig. S2 in the supplemental material). EMSAs with different amounts of purified hexahistidyl-tagged RamA fusion protein (RamA_His) showed at least weak binding of RamA_His to the musF promoter region (Fig. 5C). Even at high concentra-
tions of RamAHis, no retardation was observed with the control fragment ramBp3b, which possesses no RamA binding site (64).

To test for transcriptional regulation of the musFGI operon by RamA in vivo, a transcriptional fusion between the musF promoter region and the promoterless CAT gene was constructed in the promoter probe vector pET2 (plasmid pET2-PmusF) and transformed into C. glutamicum WT and the ramA deletion mutant C. glutamicum/H9004ramA. As controls, the empty pET2 plasmid and the plasmid pET2-PmusF-TS, which carries the truncated musF promoter region lacking TSmusF, were transformed into C. glutamicum WT. CAT activities were determined in the plasmid-carrying strains during exponential growth in minimal medium with 1% (wt/vol) glucose, 1% (wt/vol) maltose, or 1% (wt/vol) acetate. CAT activity was highest in cells of C. glutamicum WT-(pET2-PmusF) cultivated on maltose (7.6 ± 1.1 mU [mg protein]−1 for cells cultivated on glucose and maltose, respectively). Removal of TSmusF from the musF promoter region led to even lower CAT activities, with cell extracts of C. glutamicum-(pET2-PmusF-TS) showing activities between 1.5 ± 0.3 mU and 1.6 ± 0.1 mU (mg protein)−1 (Fig. 5B). In accordance with the published microarray data (57) as well as the maltose uptake rates measured here for the RamA-deficient strain C. glutamicum ΔramA, we conclude from these data that RamA acts as a transcriptional activator for the carbon source-dependent transcription of musFGI at the musF promoter identified here.

Also in C. glutamicum ΔsugR, maltose uptake rates as well as the growth rates on maltose were shown to be significantly reduced compared to those of C. glutamicum WT. This led to the assumption that SugR, the repressor of ptsG, ptsS, and ldhA (27, 55, 56), acts as an activator of the genes for the maltose uptake system. However, microarray data of C. glutamicum ΔsugR versus C. glutamicum WT (27, 56) did not reveal significant changes in the expression of the mus genes in the sugR deletion mutant strain.
Also in slot blot experiments, the intensity of the signals obtained with the probes for musK, musE, and musF for RNA samples from C. glutamicum ΔsugR and C. glutamicum WT were similar (Fig. 5A). CAT activity in cell extracts of C. glutamicum ΔsugR(pET2-PmusF) was even slightly increased, to 8.5 ± 0.7 and 8.3 ± 0.3 mU (mg protein)^{-1}, in cells cultivated on glucose and maltose, respectively (Fig. 5B). As we did not observe sequence motifs of possible SugR binding sites in the intergenic regions of the mus genes and no SugR binding sites within the mus gene cluster were identified in recently published ChIP-to-chip experiments (55), it has to be assumed that SugR is not involved in the transcriptional control of musK, musE, and musFGI. The severe effects observed on maltose uptake and metabolism in the SugR-deficient strain C. glutamicum ΔsugR, however, indicate that SugR affects expression of MusK, EFGI, the ABC transporter for maltose, by an unknown posttranscriptional mechanism.

Maltose uptake by MusK, EFGI is necessary for the positive effect of maltose on ptsG expression. In C. glutamicum, expression of ptsG is enhanced by the presence of the non-PTS substrate maltose (7, 27), which was shown here to be exclusively taken up via the ABC transporter MusK, EFGI. To distinguish whether presence of maltose in the culture broth or intermediates formed in the course of maltose metabolism give rise to this positive effect of maltose on ptsG expression, we analyzed the activity of the ptsG promoter using reporter gene assays with the promoter-probe plasmid pET2-PrptsG (27) in C. glutamicum WT and C. glutamicum Δmus cells cultivated with different carbon sources. Plasmid pET2-PrptsG contains a fusion between the ptsG promoter region and the promoterless cat gene (19). As depicted in Fig. 6, the positive effect of maltose addition on ptsG expression in C. glutamicum WT(pET2-PrptsG) led to an increase of the specific CAT activity from 0.40 ± 0.06 U (mg protein)^{-1} measured in extracts of glucose-grown cells to 0.53 ± 0.7 U (mg protein)^{-1} for extracts from cells cultivated with maltose. As previously described by Engels and Wendisch (27), the presence of acetate in the culture broth leads to significantly reduced ptsG promoter activities of 0.18 ± 0.06 and 0.19 ± 0.05 U (mg protein)^{-1} in C. glutamicum WT(pET2-PrptsG) cells cultivated with acetate and glucose plus acetate, respectively. In the maltose uptake-deficient strain C. glutamicum Δmus(pET2-PrptsG), ptsG promoter activities were nearly identical to the activities measured for C. glutamicum WT(pET2-PrptsG). Promoter activities of 0.44 ± 0.03, 0.17 ± 0.05, and 0.19 ± 0.05 U (mg protein)^{-1} were measured for C. glutamicum Δmus(pET2-PrptsG) cells cultivated with glucose, acetate, or glucose plus acetate, respectively. These data show that in both strains, expression of ptsG is repressed by the presence of acetate. As expected for C. glutamicum WT(pET2-PrptsG), maltose addition to the culture broth containing glucose plus acetate significantly increased ptsG promoter activity to 0.54 ± 0.06 U (mg protein)^{-1}, which corresponds to the increased activity measured in cells cultivated with maltose as the sole carbon source. However, for C. glutamicum Δmus(pET2-PrptsG), no positive effect on the ptsG promoter activity was detected when maltose was added to the culture broth already containing glucose plus acetate. The specific CAT activity of 0.20 ± 0.05 U (mg protein)^{-1} for C. glutamicum Δmus(pET2-PrptsG) cultured with glucose plus acetate plus maltose is about the same as the activities determined in cells cultivated with acetate or acetate plus glucose. This result clearly shows that uptake of maltose by MusK, EFGI in C. glutamicum cells is a prerequisite for the positive effect of maltose on ptsG expression.

DISCUSSION

The positive effect of maltose on expression of the ptsG-encoded EII^eff of the PTS depends on maltose uptake by the MusK, EFGI transporter identified here. It seems reasonable to suggest that intermediates of maltose metabolism trigger the positive effect on ptsG expression. In C. glutamicum, transcription of ptsG is controlled by the global regulators RamA, Ramb, GlxR, and SugR (27, 57, 65), which all coordinate the adaptation of the central metabolism of C. glutamicum toward the utilization of carbon sources requiring gluconeogenesis, as such acetate (54). Repression of ptsG in the presence of acetate is mainly caused by SugR (27). Deletion of sugR indeed abolishes the negative effects of acetic addition on ptsG expression and glucose utilization (27, 66). However, the positive effect of maltose on ptsG expression is still present in the sugR deletion mutant (27). Therefore, it is unlikely that the effect of maltose addition on ptsG expression is brought about by increased intracellular concentrations of one of the negative effectors of SugR, namely, fructose-6-phosphate and/or fructose-1-phosphate (27, 56).

The CAMP receptor protein (CRP) homologue of C. glutamicum, GlxR, acts both as a transcriptional repressor (for gltA, aceB, sdhCAB [67–69]) and transcriptional activator (for ptsSCAB, narKGHJI, catC, and atpB [70–72]) and binds in a cyclic AMP (CAMP)-dependent manner at its consensus motif, 5’-TGTGA-N_n-TCA-3’, in the promoter regions of several genes (65, 68, 72). Indeed, two GlxR binding sites are situated close to the ptsG transcriptional start site (65, 72), indicating GlxR-dependent control of ptsG expression. As neither effects of maltose addition on CAMP levels nor the role of GlxR in the transcriptional control of ptsG have been studied so far, assumptions that the positive effect of maltose addition on ptsG expression relies on changes in the CAMP levels, which might lead to GlxR-mediated activation of ptsG transcription, are hypothetical.

In reporter gene assays using the reporter plasmid pET2-PrptsG in the RamA-deficient C. glutamicum ΔramA strain, CAT activity in cells cultivated on glucose was reduced about 60%,
0.18 ± 0.02 U (mg protein)\(^{-1}\), compared to activity in \textit{C. glutamicum} WT (0.40 ± 0.06 U [mg protein]\(^{-1}\)). These data show that RamA acts as the activator of \textit{ptsG} transcription. Moreover, the positive effect of maltose on \textit{ptsG} transcription was absent from \textit{C. glutamicum} ΔramA, and CAT activity in extracts of \textit{C. glutamicum} ΔramA (pET2-PrptsG) cultivated on maltose (0.17 ± 0.02 U [mg protein]\(^{-1}\)) was identical to the activity of cells grown on glucose. These results lead to the assumption that RamA mediates the positive effect of maltose on \textit{ptsG} transcription. However, in addition to \textit{ptsG} expression, expression of \textit{musFGI}, and probably of \textit{musE}, was shown here to be activated by RamA. As a result, maltose uptake by MusK-EFGK2I is reduced in \textit{C. glutamicum} ΔramA. It is therefore complex to discriminate between the indirect involvement of RamA as an activator of \textit{musFGI} expression influencing the uptake of maltose required for the effect on \textit{ptsG} and the direct participation of RamA as a mediator of the positive effect of maltose on \textit{ptsG} expression. Although in \textit{C. glutamicum} ΔsugR the maltose uptake rate was reduced, similar to that in \textit{C. glutamicum} ΔramA, and the maltose effect on \textit{ptsG} expression was still present in \textit{C. glutamicum} ΔsugR, it seems reasonable to suggest that RamA is directly involved in the positive effect on \textit{ptsG}. Taking these results together, an intermediate of maltose metabolism probably triggers the positive effect on \textit{ptsG} expression, which most is likely mediated via RamA. As RamA effector molecules have not been identified before (57), its role in the maltose effect on \textit{ptsG} expression and, therefore, the underlying mechanism remain elusive.

Maltose uptake in \textit{C. glutamicum} is brought about by the ABC transporter system MusEFGK₁, which is unusual, as it requires an NBD dimer, a substrate binding protein (SBP), and two TMDs, which form the translocation pore (73–75). The domains of ABC importers exist in several transporters as single proteins or are arranged in other transporters in various protein fusions (76). This domain organization of ABC transporters is used for their classification (75, 76). The additional membrane protein MusL of \textit{C. glutamicum} maltose importer might be an essential accessory protein or a novel variant of the organization of an ABC transporter’s TMDs into proteins and genes. Possible homologues of MusL are the hypothetical proteins BL0145 of \textit{Bifidobacterium longum} NCC2705, SP₁₆₇₇ of \textit{Streptococcus pneumoniae} NCC2705, SP₁₆₇₇ of \textit{C. glutamicum} is not the sole ABC transport system requiring an additional membrane protein for its functionality.

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