

# Maltose Uptake by the Novel ABC Transport System MusEFGK<sub>2</sub>I Causes Increased Expression of *ptsG* in *Corynebacterium glutamicum*

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The Gram-positive *Corynebacterium glutamicum* efficiently metabolizes maltose by a pathway involving maltodextrin and glucose formation by 4- $\alpha$ -glucanotransferase, glucose phosphorylation by glucose kinases, and maltodextrin degradation via maltodextrin phosphorylase and  $\alpha$ -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*  $\Delta$ *mus* 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 [<sup>14</sup>C]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*  $\Delta$ *mus*. Indeed, maltose uptake by the novel ABC transport system MusEFGK<sub>2</sub>I is required for the positive effect of maltose on *ptsG* expression in *C. glutamicum*.

*Corynebacterium glutamicum*, a Gram-positive, nonsporulating bacterium, is widely employed in the large-scale production of amino acids, mainly L-glutamate and L-lysine (1, 2). The organism can be cultivated on a large variety of substrates, such as carbohydrates, alcohols, and organic acids (3, 4). Maltose serves as an excellent substrate for cultivation of and amino acid production with *C. glutamicum* (5, 6). Furthermore, addition of maltose to the culture broth positively affects glucose utilization by this bacterium, a unique phenomenon which can be exploited to significantly improve amino acid production (7). Based on enzyme assays and analyses of the genome sequence, a metabolic pathway for maltose utilization in *C. glutamicum* has been described (6) which involves maltodextrin and glucose formation catalyzed by the 4- $\alpha$ -glucanotransferase MalQ, maltodextrin degradation to glucose 6-phosphate via the maltodextrinphosphorylase MalP and the  $\alpha$ -phosphoglucomutase Pgm, and phosphorylation of the glucose derived from the MalQ reaction by the glucose kinases Glk and Ppgk (8, 9). Nonetheless, hitherto a maltose uptake system has not been identified in *C. glutamicum*.

Similar metabolic pathways for maltose utilization have already been identified in the Gram-negative bacterium *Escherichia coli* (reviewed in reference 10) and the archaeon *Thermococcus litoralis* (11), which both use well-characterized ATP-binding cassette (ABC) transporters for maltose uptake (12–18). However, in Gram-positive bacteria, maltose uptake is accomplished by various types of uptake systems, such as maltose-H<sup>+</sup> symporters in, e.g., *Bacillus licheniformis* (19) and *Lactobacillus sanfrancisco* (20), phosphoenolpyruvate sugar phosphotransferase systems (PTS) in, e.g., *Streptococcus bovis* (21), *Streptococcus mutans* (22), and *Bacillus subtilis* (23), or ABC transporters in, e.g., *Alicyclobacillus acidocaldarius* (24). Analyses of the genome sequence of *C. glutamicum* indicated the presence of about 260 putative transport proteins (25, 26), the majority belonging to the categories of sec-

ondary carriers and of primary transporters (117 and 106, respectively). Furthermore, four phosphotransferase system enzyme II genes are present in *C. glutamicum*.

The positive effect of maltose on glucose utilization in *C. glutamicum* comes with the increased expression of *ptsG* (7, 27), which encodes the glucose-specific EII permease (EII<sup>Glc</sup>) of the PTS (28, 29). Based on this observation, it seems reasonable to suggest that the *ptsG*-encoded EII<sup>Glc</sup> additionally catalyzes maltose uptake; however, inactivation of *ptsG* itself or any of the three further genes for EII permeases in *C. glutamicum* did not affect growth with maltose as the sole carbon source (29).

In this study, we identified the maltose/maltodextrin uptake system (MUS) of *C. glutamicum* as an ABC transporter by combination of data obtained through characterization of maltose uptake with recently published transcriptome data. This ABC sugar uptake system is unusual, as it requires an additional, uncharacterized membrane protein for maltose transport activity. Here, we show that this uptake system is essential for growth of *C. glutamicum* on maltose and maltotriose as sole carbon sources. Furthermore, we investigated the transcriptional organization of the genes encoding the maltose uptake system in *C. glutamicum* and

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TABLE 1 Strains used in this study

Strain	Relevant characteristic(s)	Reference/source
<i>E. coli</i>		
DH5 $\alpha$	<i>F<sup>-</sup> thi-1 endA1 hsdR17(r<sup>-</sup> m<sup>-</sup>) supE44 <math>\Delta</math>lacU169 (<math>\Phi</math>80lacZ<math>\Delta</math>M15) recA1 gyrA96 relA1</i>	80
<i>C. glutamicum</i>		
WT	ATCC 13032; wild-type strain	American Type Culture Collection
$\Delta$ ramB	<i>ramB</i> -negative mutant of <i>C. glutamicum</i> WT	41
$\Delta$ ramA	<i>ramA</i> -negative mutant of <i>C. glutamicum</i> WT	42
$\Delta$ sugR	<i>sugR</i> -negative mutant of <i>C. glutamicum</i> WT	27
$\Delta$ mus	In-frame deletion of genes <i>cg2708</i> to <i>cg2703</i> of <i>C. glutamicum</i> WT	This work
IMcg2708	<i>C. glutamicum</i> WT with insertion of pDrive in <i>cg2708</i>	This work
IMcg2707	<i>C. glutamicum</i> WT with insertion of pDrive in <i>cg2707</i>	This work
IMcg2705	<i>C. glutamicum</i> WT with insertion of pDrive in <i>cg2705</i>	This work
IMcg2704	<i>C. glutamicum</i> WT with insertion of pDrive in <i>cg2704</i>	This work
IMcg2701	<i>C. glutamicum</i> WT with insertion of pDrive in <i>cg2701</i>	This work
$\Delta$ ptsG	In-frame deletion of <i>ptsG</i> gene ( <i>cg1537</i> ) of <i>C. glutamicum</i> WT	This work

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 of *C. glutamicum* and *E. coli*. When appropriate, the media contained kanamycin (50  $\mu$ g ml<sup>-1</sup> for plasmids and 15  $\mu$ g ml<sup>-1</sup> for integration mutants and transformant selection) and/or chloramphenicol (25  $\mu$ g ml<sup>-1</sup>). *C. glutamicum* was grown aerobically at 30°C, and *E. coli* at 37°C, as 50-ml cultures in 500-ml baffled Erlenmeyer flasks on a rotary shaker at 120 rpm. Growth experiments with maltotetraose or maltopentaose as the substrate were performed as 10-ml cultures in 100-ml baffled Erlenmeyer

flasks. Growth of the bacteria was monitored by measuring the optical density at 600 nm (OD<sub>600</sub>). Maltose and maltotriose concentrations were analyzed by high-performance liquid chromatography (HPLC) as described previously (6).

**DNA preparation, manipulation, and transformation.** Standard procedures were employed for plasmid isolation and for molecular cloning and transformation of *E. coli*, as well as for electrophoresis (31). Isolation of chromosomal DNA and plasmids of *C. glutamicum* was performed as described previously (32). Transformation of *C. glutamicum* was performed by electroporation as described by Tauch et al. (33). PCR experiments were performed in a Flexcycler (Analytik Jena) with *Taq* DNA polymerase (MBI Fermentas) or Precisior high-fidelity DNA polymerase (BioCat GmbH) with oligonucleotides obtained from Eurofins MWG Operon. All restriction enzymes, T4-DNA ligase, and shrimp alkaline phosphatase were obtained from New England BioLabs and used according to the manufacturer's instructions.

**Construction of *C. glutamicum* mutant strains.** In-frame deletions of the genomic locus comprising *cg2708* to *cg2703* (*mus* genes) and of the *ptsG* (*cg1537*) gene were constructed using pK19*mobsacB* as described

TABLE 2 Plasmids used in this study

Plasmid	Relevant characteristic(s)	Reference/source
pK19 <i>mobsacB</i>	Kan <sup>r</sup> , mobilizable <i>E. coli</i> vector for the construction of insertion and deletion mutants in <i>C. glutamicum</i> ( <i>oriV</i> , <i>sacB</i> , <i>lacZ<math>\alpha</math></i> )	81
pK19 <i>mobsacB</i> -delMus	Kan <sup>r</sup> , pK19 <i>mobsacB</i> with the deletion construct for region <i>cg2708</i> to <i>cg2703</i>	This work
pK19 <i>mobsacB</i> -delPtsG	Kan <sup>r</sup> , pK19 <i>mobsacB</i> with the deletion construct for <i>cg1537</i>	This work
pXMJ19	Expression vector, <i>ptac</i> , <i>lacI<sup>q</sup></i> , Cm <sup>r</sup>	36
pXMJ19-musKEFG	pXMJ19 carrying region <i>cg2708</i> to <i>cg2703</i>	This work
pXMJ19-musKEFGI	pXMJ19 carrying region <i>cg2708</i> to <i>cg2701</i>	This work
pXMJ19-cg2701	pXMJ19 carrying gene <i>cg2701</i>	This work
pXMJ19-cg2701-strep	pXMJ19 carrying gene <i>cg2701</i> with sequence for C-terminal Strep-tag	This work
pJET1.2	Amp <sup>r</sup> , PCR cloning vector, P <sub>T7</sub> , <i>eco47IR</i>	Fermentas
pDrive	Amp <sup>r</sup> , Kan <sup>r</sup> , PCR cloning vector, <i>lacZ<math>\alpha</math></i> , <i>oriF1</i> , <i>ori-pUC</i>	Qiagen
pDrive-IMcg2708	Amp <sup>r</sup> , Kan <sup>r</sup> , pDrive with internal fragment of <i>cg2708</i>	This work
pDrive-IMcg2707	Amp <sup>r</sup> , Kan <sup>r</sup> , pDrive with internal fragment of <i>cg2707</i>	This work
pDrive-IMcg2705	Amp <sup>r</sup> , Kan <sup>r</sup> , pDrive with internal fragment of <i>cg2705</i>	This work
pDrive-IMcg2704	Amp <sup>r</sup> , Kan <sup>r</sup> , pDrive with internal fragment of <i>cg2704</i>	This work
pDrive-IMcg2701	Amp <sup>r</sup> , Kan <sup>r</sup> , pDrive with internal fragment of <i>cg2701</i>	This work
pDrive-RACE-cg2704	Amp <sup>r</sup> , Kan <sup>r</sup> , pDrive carrying the PCR-amplified fragment from the <i>cg2704</i> RACE assay	This work
pET2	Promoter probe vector carrying the promoterless <i>cat</i> gene, Km <sup>r</sup>	82
pET2-PR <i>musF</i>	pET2 containing the <i>musF</i> promoter fragment	This work
pET2-PR <i>musF</i> -TS	pET2 containing a truncated <i>musF</i> promoter fragment	This work
pET2-PR <i>ptsG</i>	pET2 containing the <i>ptsG</i> promoter fragment	27

previously (34). In detail, the flanking regions of the gene/cluster were amplified using primer pairs  $\Delta$ -gene/cluster\_1 plus  $\Delta$ -gene/cluster\_2 and  $\Delta$ -gene/cluster\_3 plus  $\Delta$ -gene/cluster\_4 (see Table S1 in the supplemental material). The two flanking PCR products obtained served as the templates for a crossover PCR using the primer pair  $\Delta$ -gene/cluster\_1 plus  $\Delta$ -gene/cluster\_4. The resulting PCR product was digested with the enzymes indicated in Table S1 and cloned into pK19*mobsacB* cut with the same enzymes. Gene deletion with the derived pK19*mobsacB* $\Delta$ 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 $\Delta$ MusFOR and check $\Delta$ MusREV, resulting in a 7,018-bp fragment for the wild type (WT) and a 1,188-bp fragment for the *mus* deletion mutant; *ptsG* deletion was verified using the primers check $\Delta$ ptsGFOR and check $\Delta$ ptsGREV, 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 supplemental 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 isopropyl- $\beta$ -D-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 supplemental 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* DH5 $\alpha$ . 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 PR*musF*-for and PR*musF*-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-*PmusF*. 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 PR*musF*-TS-for and PR*musF*-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 MgCl<sub>2</sub> and 1 mM EDTA. The specific CAT activity was determined as described by Schreiner et al. (38). Protein concentrations were determined using the Roti-Nanoquant kit (Roth) with bovine serum albumin as the standard. SDS-PAGE was performed according to Laemmli (39). Loading buffer (4 $\times$ ) contained 8% (wt/vol) SDS, 20% (vol/vol) glycerol, 10 mM EDTA, 100 mM Tris-HCl, pH 6.8, 2% (vol/vol)  $\beta$ -mercaptoethanol, and 1 mg/ml bromophenol blue. Membrane preparations and Western blot experiments for detection of the Streptavidin-tagged Cg2701 protein by using antibodies raised against the Strep-tag 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 Ni<sup>2+</sup> affinity chromatogra-

phy 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 PR*musF*-for and PR*musF*-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  $\mu$ g) in 20  $\mu$ l 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  $\mu$ g poly[d(I·C)] for 20 min at room temperature. Subsequently, the mixture was separated on a 2% agarose gel in 1  $\times$  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 supplemental 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<sup>-2</sup>. 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  $\mu$ g of total RNA using the gene-specific primer designated RACE-*cg2704*-SP1 in Table S1 in the supplemental 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.

**[<sup>14</sup>C]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 OD<sub>600</sub> 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  $\mu$ M to 1 mM [<sup>14</sup>C]maltose (specific activity, 679 mCi  $\mu$ mol<sup>-1</sup>; 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- $\mu$ l 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 (Rotiszint; 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 rho-independent transcriptional terminators was performed using TransTermHP (47), and MFold (48) was the software used for the calculation of their  $\Delta G^{o'}$  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  $\mu\text{M}$ ) 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  $1.0 \pm 0.2 \mu\text{M}$  and a  $V_{\text{max}}$  of  $22.6 \pm 0.8 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ cell dry matter (cdm)}$  (see Fig. S1 in the supplemental material). Uptake of maltose in cells of the EII<sup>Glc</sup>-deficient strain *C. glutamicum*  $\Delta\text{ptsG}$  cultivated on TY complex medium proceeded with a rate of  $27.8 \pm 1.8 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ cdm}$ , which is nearly identical to that of cells of *C. glutamicum* WT ( $29.4 \pm 2.0 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ cdm}$ ), when cells were cultivated in TY and measurements were performed at a maltose concentration of 100  $\mu\text{M}$ . 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  $\mu\text{M}$  maltose. Simultaneous addition of valinomycin (ionophore for  $\text{K}^+$ ) and nigericin (ionophore for  $\text{H}^+$  and  $\text{K}^+$ ) to *C. glutamicum* cells abolishes the proton-motive force necessary to drive secondary active transporters (52, 53). However, maltose uptake was only reduced to  $17.0 \pm 5.0 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ cdm}$  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* ( $11.0 \pm 5.0 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ cdm}$ ; 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 ( $17.0 \pm 6.4$  and  $22.7 \pm 3.4 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ cdm}$ , respectively). Reduced [ $^{14}\text{C}$ ]maltose uptake rates were observed for cells cultivated on lactate ( $13.3 \pm 1.5 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ cdm}$ ), on fructose ( $10.7 \pm 1.7 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ cdm}$ ), or on acetate ( $10.7 \pm 1.0 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ cdm}$ ). 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*.

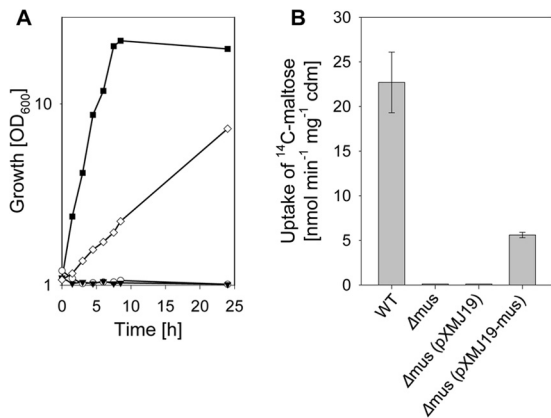
Three transcriptional regulators, RamA, RamB, and SugR, have been shown to be master regulators for the adjustment of the central metabolism of *C. glutamicum* in response to the utilization of the gluconeogenic substrate acetate (reviewed in reference 54). We therefore analyzed [ $^{14}\text{C}$ ]maltose uptake and maltose utilization by the RamA-deficient strain *C. glutamicum*  $\Delta\text{ramA}$ , the RamB-deficient strain *C. glutamicum*  $\Delta\text{ramB}$ , and the SugR-deficient strain *C. glutamicum*  $\Delta\text{sugR}$ . As both uptake of ( $9.1 \pm 0.3 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ cdm}$ ) and growth on (growth rate of  $0.27 \pm 0.01 \text{ h}^{-1}$ ) maltose were significantly reduced in *C. glutamicum*  $\Delta\text{ramA}$  compared to *C. glutamicum* WT (uptake rate,  $30.3 \pm 2.4 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ cdm}$ ; growth rate,  $0.37 \pm 0.03 \text{ h}^{-1}$ ), we concluded that RamA probably acts as an activator of genes for maltose

utilization/uptake. Maltose uptake ( $10.8 \pm 0.4 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ cdm}$ ) and growth on maltose ( $0.27 \pm 0.02 \text{ h}^{-1}$ ) were also found to be slowed in *C. glutamicum*  $\Delta\text{sugR}$ . 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*  $\Delta\text{ramB}$  in minimal medium with maltose ( $0.34 \pm 0.02 \text{ h}^{-1}$ ) as well as maltose uptake ( $18.9 \pm 1.5 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ cdm}$  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*  $\Delta\text{ramA}$ , *C. glutamicum*  $\Delta\text{ramB}$ , and *C. glutamicum*  $\Delta\text{sugR}$ , 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, preferentially 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*  $\Delta\text{ramA}$  when cultivated on glucose, (iv) be repressed in *C. glutamicum*  $\Delta\text{sugR}$ , and (v) be slightly affected in the *ramB* deletion mutant *C. glutamicum*  $\Delta\text{ramB}$ . 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*  $\Delta\text{ramA}$  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*  $\Delta\text{mus}$ , 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*  $\Delta\text{mus}$ . 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*  $\Delta\text{mus}$  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*).

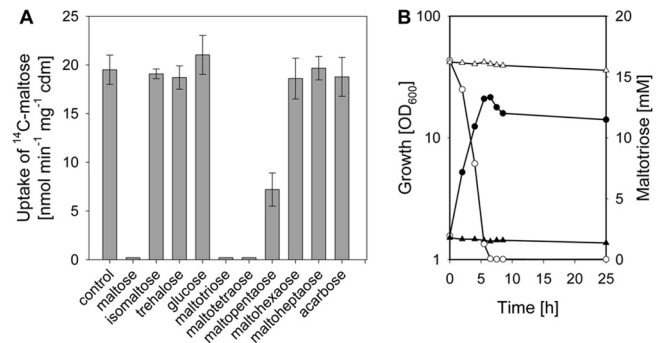
**Maltodextrin uptake by the *musKEFG*-encoded ABC transport system.** To analyze the substrate specificity of the ABC trans-



**FIG 1** Growth (A) and rates of [<sup>14</sup>C]maltose uptake (B) of *C. glutamicum* WT (filled squares), *C. glutamicum*  $\Delta mus$  (open circles), *C. glutamicum*  $\Delta mus(pXMJ19)$  (filled triangles), and *C. glutamicum*  $\Delta mus(pXMJ19-musKEFG)$  (open diamonds) in CgC minimal medium initially containing 2% (wt/vol) maltose. One representative growth curve of at least three independent cultivations is shown in panel A; the results of each of the cultivations were comparable. For uptake measurements, the strains were cultivated in CgC minimal medium with 2% (wt/vol) glucose. The data represent mean values and standard deviations of three independent measurements from three independent cultivations.

porter encoded by the *musKEFG* genes, [<sup>14</sup>C]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 [<sup>14</sup>C]maltose uptake were observed by addition of glucose or the disaccharides trehalose and isomaltose. Addition of the maltodextrins maltotriose and maltotetraose completely quenched the uptake of [<sup>14</sup>C]maltose; furthermore, maltopentaose reduced the [<sup>14</sup>C]maltose uptake rate from  $19.5 \pm 1.5$  nmol min<sup>-1</sup> mg<sup>-1</sup> cdm to  $6.8 \pm 0.3$  nmol min<sup>-1</sup> mg<sup>-1</sup> 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.

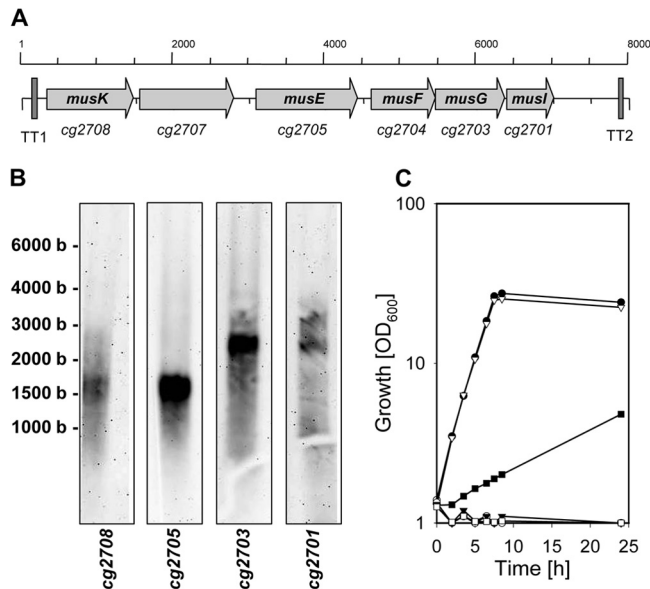
However, the assay utilized here does not discriminate between competition for transport or only binding. To analyze if short maltodextrins are indeed substrates of the *musKEFG*-encoded ABC transport system, growth experiments with *C. glutamicum* WT and *C. glutamicum*  $\Delta mus$  in minimal medium with maltotriose as the sole carbon source were performed. As shown in Fig. 2B, *C. glutamicum* utilized maltotriose as a carbon source, grew at a rate of  $0.38 \pm 0.02$  h<sup>-1</sup>, and reached a final optical density of  $14.0 \pm 0.5$  after 24 h of cultivation. Neither growth nor maltotriose utilization was observed for *C. glutamicum*  $\Delta mus$  (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  $0.34 \pm 0.02$  h<sup>-1</sup> and a final optical density of  $10.5 \pm 0.5$  were measured. No growth on maltotetraose was observed for *C. glutamicum*  $\Delta mus$ . In growth experiments with 0.5% (wt/vol) maltopentaose as the sole substrate,



**FIG 2** Effect of unlabeled competitors on [<sup>14</sup>C]maltose uptake rates in *C. glutamicum* (A) and growth (filled symbols) and substrate consumption (open symbols) of *C. glutamicum* WT (circles) and *C. glutamicum*  $\Delta mus$  in CgC minimal medium with 1% (wt/vol) maltotriose as the sole carbon source (B). One representative growth curve of at least three independent cultivations is shown; the results of each of the cultivations were comparable. The uptake measurements were started by simultaneous addition of 50  $\mu$ M [<sup>14</sup>C]maltose and 5 mM competitor. As a control, [<sup>14</sup>C]maltose uptake was measured without competitor. Cells for the experiments were cultivated in CgC minimal medium with 1% (wt/vol) glucose. The data represent mean values and standard deviations of three independent measurements from two independent cultivations.

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;  $\Delta G^{\circ}$ ,  $-21.9$  kcal mol<sup>-1</sup>; 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*;  $\Delta G^{\circ}$ ,  $-13.6$  kcal mol<sup>-1</sup>; 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



**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 triangles), *C. glutamicum* IMcg2705 (filled triangles), *C. glutamicum* IMcg2703 (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.

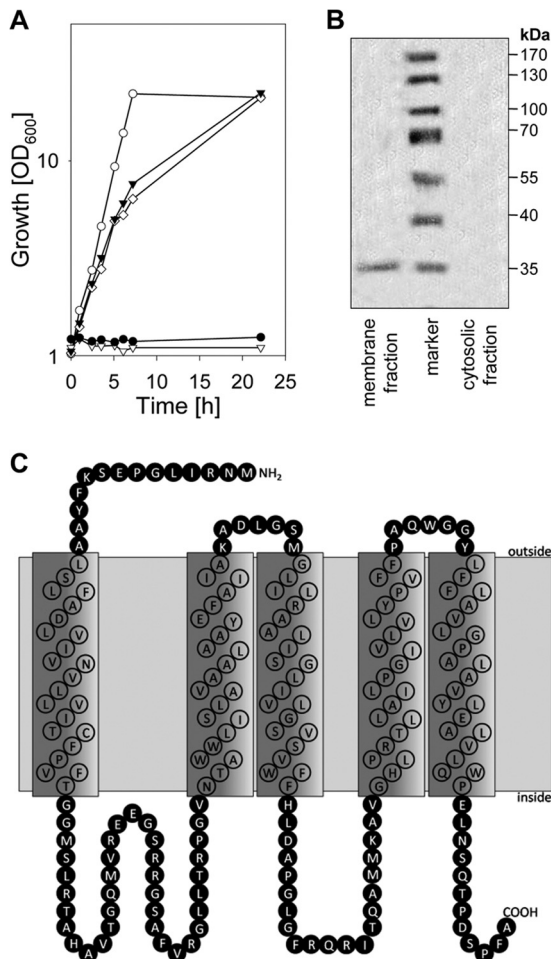
shown). In three independent experiments, the transcriptional start site of *musF* ( $TS_{musF}$ ) was found to be a cytosine residue within the *musE*-*musF* intergenic region, located 64 bp upstream of the *musF* ATG start codon and 122 bp downstream of the *musE* TAA stop codon (see Fig. S2 in the supplemental material). Upstream of  $TS_{musF}$  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* (CGTCCT [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* IM*musK*, *C. glutamicum* IM*cg2707*, *C. glutamicum* IM*musE*, *C. glutamicum* IM*musF*, and *C. glutamicum* IM*cg2701*, 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  $^{14}\text{C}$ -labeled maltose was also abolished in both *C. glutamicum* IM*musE* and *C. glutamicum* IM*musF* (results not shown). As both utilization of maltose as the sole carbon source (Fig. 3C) and uptake of [ $^{14}\text{C}$ ]maltose were slowed down at least 5-fold in *C. glutamicum* IM*musK* ( $2.5 \pm 0.5 \text{ nmol min}^{-1} \text{ mg}^{-1}$

$\text{cdw}$ ; growth rate,  $0.07 \pm 0.5 \text{ h}^{-1}$ ) compared to the parental strain *C. glutamicum* WT ( $22.7 \pm 3.4 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ cdm}$ ; growth rate,  $0.37 \pm 0.01 \text{ h}^{-1}$ ), we conclude that an alternative, unidentified ATPase can partially replace MusK. Inactivation of the ORF *cg2707*, which encodes a hypothetical soluble protein, did not affect maltose utilization (growth rate,  $0.36 \pm 0.02 \text{ h}^{-1}$ ) (Fig. 3C) and uptake of maltose (maltose uptake rate for *C. glutamicum* IM*cg2707* of  $27.5 \pm 2.8 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ cdm}$ ). 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* IM*cg2701*. 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* IM*cg2701* 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* IM*cg2701* 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  $^{14}\text{C}$ -labeled maltose was observed for *C. glutamicum* IM*cg2701*(pXMJ19-*cg2701*) (maltose uptake rate,  $16.7 \pm 2.3 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ cdm}$ ; results not shown), while no uptake of label was detected for *C. glutamicum* IM*cg2701* (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 ( $17.1 \pm 1.9 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ cdm}$ ) in *C. glutamicum* IM*cg2701*. 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* IM*cg2701*(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*  $\Delta\text{mus}$  using plasmid pXMJ19-*mus*, as this plasmid only harbors the genes *musK* to *musG* 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-*musEFGKI* in *C. glutamicum*  $\Delta\text{mus}$ , did not further improve growth on maltose, as identical growth rates for *C. glutamicum*  $\Delta\text{mus}$ (pXMJ19-*musKEFGI*) and *C. glutamicum*  $\Delta\text{mus}$ (pXMJ19-*musEFGK*) were observed ( $0.26 \pm 0.02 \text{ h}^{-1}$  and  $0.27 \pm 0.03 \text{ h}^{-1}$ , 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*  $\Delta\text{mus}$ (pXMJ19-*musKEFG*) 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*  $\Delta\text{mus}$  and *C. glutamicum*  $\Delta\text{mus}$ (pXMJ19-*musKEFG*) (data not shown). Indeed, stronger signals for *cg2701* expression were detected for the *C. glutamicum* WT and for *C. glutamicum*  $\Delta\text{mus}$ (pXMJ19-*musKEFGI*), which carries the plasmid comprising the complete *mus* gene clus-



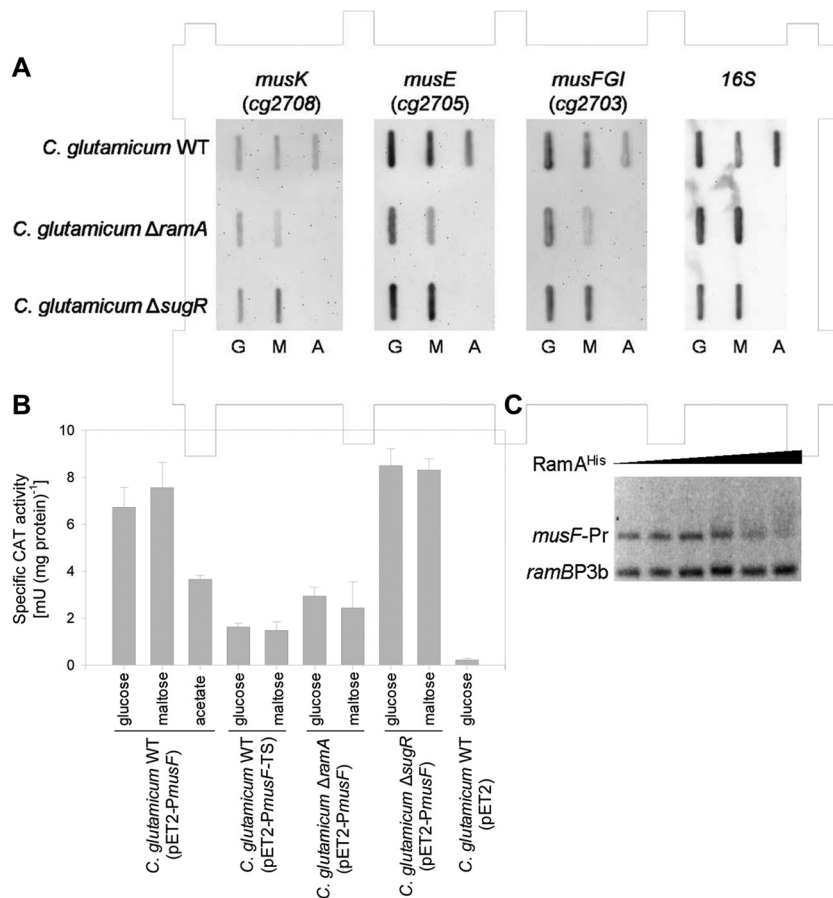
**FIG 4** Growth of *C. glutamicum* WT (open circles), *C. glutamicum* IMcg2701 (open triangles), *C. glutamicum* IMcg2701(pXMJ19-cg2701) (filled triangles), *C. glutamicum* IMcg2701(pXMJ19-cg2701-strep) (open squares), and *C. glutamicum* IMcg2701(pXMJ19) (filled circles) in CgC minimal medium with 2% (wt/vol) maltose (A), and topology model of MusI (C). For localization of MusI-Strep, *C. glutamicum* IMcg2701(pXMJ19-cg2701-strep) cells were harvested, washed, and disrupted. Cytosolic and membrane fractions separated by ultracentrifugation were then electrophoresed on a 12% SDS-polyacrylamide gel and transferred to a membrane. MusI-Strep was detected using anti-Strep antibody. A PAGE ruler prestained protein ladder (MBI Fermentas) was used as the marker. The topology model of the membrane-spanning regions of MusI is based on topology modeling. Membrane-spanning sequences were determined based on consensus sequences from two topology prediction algorithms. One representative growth curve from at least three independent cultivations is shown. The results of each of the cultivations were comparable.

ter. These data show that although the *musFG-cg2701* operon is disrupted, weak transcription of *cg2701* from an unknown promoter takes place which is sufficient to support growth of *C. glutamicum*  $\Delta$ *mus*(pXMJ19-musKEFG) on maltose.

As described before, in addition to maltose, maltotriose and maltotetraose also are taken up via the *musKEFG*-encoded ABC transporter. No growth of *C. glutamicum* IMcg2701 in minimal medium with maltotriose or maltotetraose as the sole source of carbon and energy was observed. The *cg2701*-encoded membrane protein therefore is also necessary for the uptake of short malto-

dextrins. 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*  $\Delta$ *ramA* and the SugR-deficient strain *C. glutamicum*  $\Delta$ *sugR* 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*  $\Delta$ *ramA*, and *C. glutamicum*  $\Delta$ *sugR* 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*  $\Delta$ *ramA* showed that fewer transcripts of *musE* and *musF* were present in the mutant 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*  $\Delta$ *ramA* 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 TS<sub>musF</sub>, 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<sup>His</sup>) showed at least weak binding of RamA<sup>His</sup> to the *musF* promoter region (Fig. 5C). Even at high concentra-



**FIG 5** (A) Representative RNA hybridization experiments with RNA isolated from *C. glutamicum* WT, *C. glutamicum*  $\Delta ramA$ , and *C. glutamicum*  $\Delta sugR$  cultivated in minimal medium with 2% (wt/vol) of the indicated carbon sources. The RNA levels of *musK*, *musE*, *musFGI*, and 16S were monitored with DIG-labeled antisense RNA probes. The results of one representative experiment from a series of three independent experiments is shown. (B) Expression of a *musF*<sup>-</sup>-*cat* fusion in *C. glutamicum* WT (pET2-*PmusF*), *C. glutamicum*  $\Delta ramA$  (pET2-*PmusF*), and *C. glutamicum*  $\Delta sugR$  (pET2-*PmusF*). As controls, CAT activity was analyzed in *C. glutamicum* WT (pET2-*PmusF*-TS), which lacks the *musF* transcriptional start site, and *C. glutamicum* WT (pET2), which carries the promoterless *cat* gene. The reporter gene activity was determined in cell extracts from cultivations in CgC minimal medium with 1% (wt/vol) of each of the indicated carbon sources. The values represent averages and standard deviations from at least three independent experiments. (C) Representative EMSA using hexahistidyl-tagged RamA protein (0 to 7.5  $\mu$ g) with the 380-bp *musF*-Pr fragment as a probe (20 ng) and the 211-bp *ramBp3b* fragment as a negative control (10 ng).

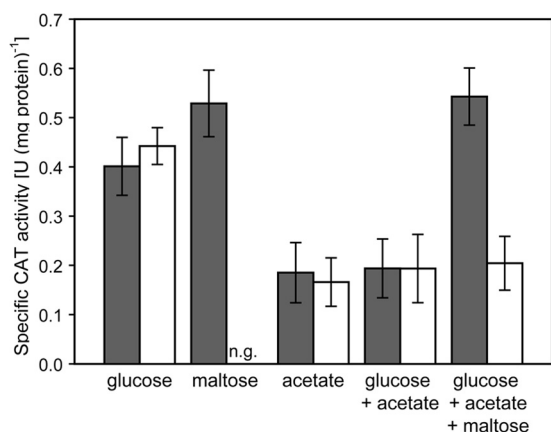
tions of RamA<sup>His</sup>, no retardation was observed with the control fragment *ramBp3b*, which possesses no RamA binding site (64).

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*  $\Delta ramA$ . As controls, the empty pET2 plasmid and the plasmid pET2-*PmusF*-TS, which carries the truncated *musF* promoter region lacking TS<sub>*musF*</sub>, 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 ( $7.6 \pm 1.1$  mU [mg protein]<sup>-1</sup>), similarly high in cells cultivated on glucose ( $6.7 \pm 0.9$  mU [mg protein]<sup>-1</sup>), and significantly reduced in cells cultivated on acetate ( $3.6 \pm 0.2$  mU [mg protein]<sup>-1</sup>) (Fig. 5B). Independent of the carbon source, the *musF* promoter activities were 60 to 80%

lower in *C. glutamicum*  $\Delta ramA$  ( $2.9 \pm 0.4$  and  $2.4 \pm 1.1$  mU [mg protein]<sup>-1</sup> for cells cultivated on glucose and maltose, respectively). Removal of TS<sub>*musF*</sub> from the *musF* promoter region led to even lower CAT activities, with cell extracts of *C. glutamicum*- (pET2-*PmusF*-TS) showing activities between  $1.5 \pm 0.3$  mU and  $1.6 \pm 0.1$  mU (mg protein)<sup>-1</sup> (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*  $\Delta ramA$ , 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*  $\Delta sugR$ , 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*  $\Delta sugR$  versus *C. glutamicum* WT (27, 56) did not reveal significant changes in the expression of the *mus* genes in the *sugR* deletion mutant strain.





**FIG 6** Expression of a *ptsG'*-*cat* fusion in *C. glutamicum* WT(pET2-*ptsG*) (dark bars) and *C. glutamicum*  $\Delta$ mus(pET2-*ptsG*) (white bars). The reporter gene activity was determined in cell extracts from cultivations in CgC minimal medium with 1% (wt/vol) of each of the indicated carbon sources. The values represent averages and standard deviations from at least three independent experiments.

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*  $\Delta$ sugR and *C. glutamicum* WT were similar (Fig. 5A). CAT activity in cell extracts of *C. glutamicum*  $\Delta$ sugR(pET2-*PmusF*) was even slightly increased, to  $8.5 \pm 0.7$  and  $8.3 \pm 0.3$  mU (mg protein)<sup>-1</sup>, 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 *musF*. The severe effects observed on maltose uptake and metabolization in the SugR-deficient strain *C. glutamicum*  $\Delta$ sugR, however, indicate that SugR affects expression of MusK<sub>2</sub>EFGI, the ABC transporter for maltose, by an unknown posttranscriptional mechanism.

**Maltose uptake by MusK<sub>2</sub>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<sub>2</sub>EFGI. To distinguish whether presence of maltose in the culture broth or intermediates formed in the course of maltose metabolization 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*  $\Delta$ mus 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  $0.40 \pm 0.06$  U (mg protein)<sup>-1</sup> measured in extracts of glucose-grown cells to  $0.53 \pm 0.7$  U (mg protein)<sup>-1</sup> 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  $0.18 \pm 0.06$  and  $0.19 \pm 0.05$  U (mg protein)<sup>-1</sup> in *C. glutamicum* WT(pET2-PRptsG) cells cultivated with acetate and

glucose plus acetate, respectively. In the maltose uptake-deficient strain *C. glutamicum*  $\Delta$ mus(pET2-PRptsG), *ptsG* promoter activities were nearly identical to the activities measured for *C. glutamicum* WT(pET2-PRptsG). Promoter activities of  $0.44 \pm 0.03$ ,  $0.17 \pm 0.05$ , and  $0.19 \pm 0.05$  U (mg protein)<sup>-1</sup> were measured for *C. glutamicum*  $\Delta$ mus(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  $0.54 \pm 0.06$  U (mg protein)<sup>-1</sup>, which corresponds to the increased activity measured in cells cultivated with maltose as the sole carbon source. However, for *C. glutamicum*  $\Delta$ mus(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  $0.20 \pm 0.05$  U (mg protein)<sup>-1</sup> for *C. glutamicum*  $\Delta$ mus(pET2-PRptsG) cultivated 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<sub>2</sub>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<sup>Glc</sup> of the PTS depends on maltose uptake by the MusK<sub>2</sub>EFGI transporter identified here. It seems reasonable to suggest that intermediates of maltose metabolization 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, such as 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 acetate 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 *pstSCAB*, *narKGGHJI*, *ctaC*, and *atpB* [70–72]) and binds in a cyclic AMP (cAMP)-dependent manner at its consensus motif, 5'-TGTGA-N<sub>6</sub>-TCACA-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*  $\Delta$ ramA strain, CAT activity in cells cultivated on glucose was reduced about 60%, to

$0.18 \pm 0.02$  U (mg protein) $^{-1}$ , compared to activity in *C. glutamicum* WT ( $0.40 \pm 0.06$  U [mg protein] $^{-1}$ ). These data show that RamA acts as the activator of *ptsG* transcription. Moreover, the positive effect of maltose on *ptsG* transcription was absent from *C. glutamicum*  $\Delta$ ramA, and CAT activity in extracts of *C. glutamicum*  $\Delta$ ramA(pET2-PRptsG) cultivated on maltose ( $0.17 \pm 0.02$  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 *ptsG* transcription. However, in addition to *ptsG* expression, expression of *musFGI*, and probably of *musE*, was shown here to be activated by RamA. As a result, maltose uptake by MusK<sub>2</sub>EFGI is reduced in *C. glutamicum*  $\Delta$ ramA. It is therefore complex to discriminate between the indirect involvement of RamA as an activator of *musFGI* expression influencing the uptake of maltose required for the effect on *ptsG* and the direct participation of RamA as a mediator of the positive effect of maltose on *ptsG* expression. Although in *C. glutamicum*  $\Delta$ sugR the maltose uptake rate was reduced, similar to that in *C. glutamicum*  $\Delta$ ramA, and the maltose effect on *ptsG* expression was still present in *C. glutamicum*  $\Delta$ sugR, it seems reasonable to suggest that RamA is directly involved in the positive effect on *ptsG*. Taking these results together, an intermediate of maltose metabolism probably triggers the positive effect on *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 *ptsG* expression and, therefore, the underlying mechanism remain elusive.

Maltose uptake in *C. glutamicum* is brought about by the ABC transporter system MusEFGK<sub>2</sub>I, which is unusual, as it requires the additional membrane protein MusI to be functional. In general, ABC importers are well understood on the mechanistic level and share a modular organization, which comprises 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 MusI of the *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 MusI are the hypothetical proteins BL0145 of *Bifidobacterium longum* NCC2705, SP\_1677 of *Streptococcus pneumoniae* TIGR4, and SAG0038 of *Streptococcus agalactiae* 2603. Although the amino acid sequences of the MusI homologues share only low similarities (see Fig. S3 in the supplemental material), each of the three hypothetical proteins consists of about 210 amino acids and contains five transmembrane stretches and the DUF (domain of unknown function) 624 motif, and it is located in the vicinity of genes for ABC importers (see Fig. S4), as is also the case for MusI. In *B. longum*, the ORF *bl0145* is directly following *bl0143* and *bl0144*, which encode TMDs of the maltose ABC importer (77). In *S. agalactiae* 2603, the ORF *sag0038* is separated by one ORF (*sag0037*), encoding a further hypothetical protein, from the ORFs *sag0034*, *sag0035*, and *sag0036*, which encode the SBP and the TMDs of the NanT ABC importer for sialic acid (78). Three ORFs (*sp1680*, *sp1679*, and *sp1678*) are situated between the genes *satA*, *satB*, and *satC* for the sialic acid ABC importer (79) and the ORF *sp1677*, which encodes the MusI-like protein (see Fig. S4). The relevance of these MusI-like proteins for substrate uptake by the aforementioned ABC importers has not been studied. As is the

case for MusF and MusG of *C. glutamicum*, based on comparisons of the amino acid sequences of the TMDs, none of the general features necessary for the functionality of ABC transporters are lacking in the *B. longum* maltose importer or the sialic acid importers of *S. agalactiae* and *S. pneumoniae*. Even though no function can be assigned to MusI or the MusI-like proteins of other ABC importers so far, the occurrence of genes for MusI-like proteins in the vicinity of several genes for ABC importers indicates that MusEFGK<sub>2</sub>I of *C. glutamicum* is not the sole ABC transport system requiring an additional membrane protein for its functionality.

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