

## Catabolite Repression and Induction of the $Mg^{2+}$ -Citrate Transporter CitM of *Bacillus subtilis*

JESSICA B. WARNER,<sup>1</sup> BASTIAAN P. KROM,<sup>1</sup> CHRISTIAN MAGNI,<sup>1,2</sup>  
WIL N. KONINGS,<sup>1</sup> AND JUKE S. LOLKEMA<sup>1\*</sup>

Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, 9751 NN Haren, The Netherlands,<sup>1</sup> and Instituto de Biología Molecular y Celular de Rosario (IBR-CONICET) and Departamento de Microbiología, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, 2000 Rosario, Argentina<sup>2</sup>

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**In *Bacillus subtilis* the *citM* gene encodes the  $Mg^{2+}$ -citrate transporter. A target site for carbon catabolite repression (*cre* site) is located upstream of *citM*. Fusions of the *citM* promoter region, including the *cre* sequence, to the  $\beta$ -galactosidase reporter gene were constructed and integrated into the *amyE* site of *B. subtilis* to study catabolic effects on *citM* expression. In parallel with  $\beta$ -galactosidase activity, the uptake of  $Ni^{2+}$ -citrate in whole cells was measured to correlate *citM* promoter activity with the enzymatic activity of the CitM protein. In minimal media, CitM was only expressed when citrate was present. The presence of glucose in the medium completely repressed *citM* expression; repression was also observed in media containing glycerol, inositol, or succinate-glutamate. Studies with *B. subtilis* mutants defective in the catabolite repression components HPr, Crh, and CcpA showed that the repression exerted by all these medium components was mediated via the carbon catabolite repression system. During growth on inositol and succinate, the presence of glutamate strongly potentiated the repression of *citM* expression by glucose. A reasonable correlation between *citM* promoter activity and CitM transport activity was observed in this study, indicating that the  $Mg^{2+}$ -citrate uptake activity of *B. subtilis* is mainly regulated at the transcriptional level.**

Carbon catabolite repression (CCR) of many genes in *Bacillus subtilis* is caused by the availability of glucose or other rapidly metabolized carbon sources during growth. The regulatory process involves several proteins including HPr, Crh, HPr kinase, and CcpA. The HPr protein, encoded by the *ptsH* gene, functions in CCR as well as in the phosphoenolpyruvate-sugar phosphotransferase system (PTS). Crh (catabolite repression HPr) is a protein homologous to HPr that functions in CCR but not in the PTS (13, 29). After glucose is taken up via the PTS, glycolytic intermediates such as fructose-1,6-bisphosphate activate HPr kinase (14, 19, 25). Then, HPr kinase phosphorylates HPr (14, 36) and Crh (13, 14) at a serine residue (Ser46) in an ATP-dependent manner. The seryl-phosphorylated proteins act as corepressors (11, 20, 24) by forming a complex (6) with the *trans*-acting CcpA (catabolite control protein A), a member of the LacI/GalR family of regulatory proteins (17). The complex binds to a consensus DNA sequence, the so-called *cre* site (catabolite-responsive element) (18), located upstream of the target gene, where it may act either as a repressor or as an activator of transcription (18).

The key role of CcpA in carbon regulation in *B. subtilis* has been demonstrated by inactivating the *ccpA* gene, which resulted in relief of glucose catabolite repression (11, 17) or the abolition of gene activation (34, 40). Mutating Ser46 of HPr resulted in relief from CCR of *gnt* (7, 31), in partial relief of *lev* (13) and *xynB* (12), and no relief of *hut* (47) and *idh* (13). In the last two cases, complete relief was observed in a *ptsHI crh* double mutant, in which HPr is mutated and the *crh* gene is disrupted. This suggests an active role for both P-Ser46-HPr

and P-Ser46-Crh in CCR, but the individual roles of the two proteins remain obscure.

Uptake of citrate in *B. subtilis* is strongly enhanced in the presence of divalent metal ions (2). At least two secondary transport proteins, the paralogues CitM and CitH, encoded by open reading frames *yflO* and *yxiQ*, respectively (26), mediate citrate uptake in *B. subtilis* (3). The transporters were expressed in *E. coli* and functionally characterized (3). CitM turned out to be the transporter that is likely to be responsible for enhanced uptake in the presence of divalent metal ions: CitM transports citrate in a complex with  $Mg^{2+}$  and several other divalent metal ions (2; B. P. Krom, J. B. Warner, W. N. Konings, and J. S. Lolkema, submitted for publication).

The structural gene coding for CitM is organized in an operon-like structure (Fig. 1), including *citM* and a second gene, *yflN* (26), the function of which is not known. Upstream of *citM* are reading frames *citS* and *citT*, which code for a two-component system (9). The CitS-CitT two-component system is essential for the transcription of the *citM-yflN* operon. The putative CitT target sequence is believed to be located in the region between nucleotides -62 and -113, upstream of the *citM* transcriptional start point (H. Yamamoto, M. Murata, and J. Sekiguchi, Abstr. 10th Int. Conf. Bacilli, p. 71, 1999). In addition, just in front of the *citM* gene lies a sequence that matches the consensus sequence of a *cre* site (41, 46). The functionality of the *cre* site has been demonstrated in vivo (32). The location of the *citM* gene on the chromosome of *B. subtilis* suggests that expression of CitM might be under the control of the metabolic state of the cell.

Induction by citrate and inhibition by glucose of citrate uptake in *B. subtilis* have been described already 3 decades ago (42). In this study we report on the regulation of synthesis of the  $Mg^{2+}$ -citrate transporter of *B. subtilis*, encoded by the *citM* gene. Transcription of *citM* is strictly dependent on the pres-

\* Corresponding author. Mailing address: Department of Microbiology, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands. Phone: 3150-3632155. Fax: 3150-3632154. E-mail: j.s.lolkema@biol.rug.nl.

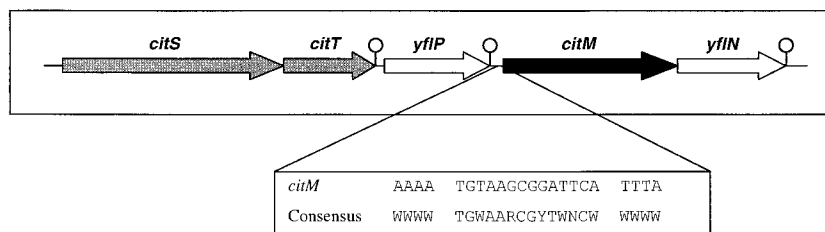


FIG. 1. Genetic organization of the *citM* gene on the *B. subtilis* chromosome. Arrows, direction of transcription; loops, transcription termination sites. *citS* and *citT* code for the two-component signal transduction system, *citM* codes for the secondary transporter of the  $Mg^{2+}$ -citrate complex, and *yfIP* and *yfIN* code for unknown proteins. The genes are drawn to scale. Below is shown the alignment of the *cre* site located in the *CitM* promoter region and the consensus sequence as described previously (48). The *citM* *cre* site is centered at  $-24$  bp relative to the start codon of the *citM* gene. Symbols for nucleotides in the consensus sequence: W, A or T; R, A or G; Y, C or T; N, A, G, C, or T.

ence of citrate in the growth medium and is under the control of CCR. Repression was observed in media containing several carbohydrates but also nonsugars. Experiments with wild-type and CCR mutant strains revealed the involvement of the different CCR components.

#### MATERIALS AND METHODS

**Bacterial strains, media, and growth conditions.** The *B. subtilis* strains used in this study are listed in Table 1. *B. subtilis* was grown in C medium (1) in which ferric ammonium citrate was omitted. The C medium was supplemented with 10 mM trisodium citrate (CC medium) or 25 mM *myo*-inositol (CI medium) or 25 mM *myo*-inositol and 8 g of potassium glutamate/liter (CIE medium), or 6 g of sodium succinate and 8 g of potassium glutamate/liter (CSE medium). When strain QB5407 was grown in CI medium, 10 mM potassium glutamate was added as nitrogen source (10). Glucose or trisodium citrate was sometimes added at a concentration of 10 mM. Auxotrophic requirements were added at 20- $\mu$ g/ml final concentration. When appropriate, antibiotics were added at concentrations of 100  $\mu$ g/ml for spectinomycin (strains QB7097 and QB5407) and 5  $\mu$ g/ml for kanamycin (QB7102) and chloramphenicol (the *PcitM-lacZ* fusion strains).

Overnight cultures of wild-type and mutant *B. subtilis* strains were inoculated into 20 ml of medium. The cells were grown in 100-ml flasks at 37°C on a rotary shaker operated at 150 rpm. Growth was monitored by measuring the optical density of the cultures at 660 nm ( $OD_{660}$ ) using a Hitachi U-1100 spectrophotometer. The cells were harvested by centrifugation in the exponential growth phase at an  $OD_{660}$  between 0.3 and 0.6 and washed once with 50 mM PIPES (piperazine-*N,N'*-bis[2-ethanesulfonic acid]), pH 6.5.

**Construction of *PcitM-lacZ* fusions.** Vector pJM116 (5) contains the promoterless *spoVG-lacZ* gene between two fragments of the *B. subtilis amyE* gene and carries the *cat* gene from pC194 (4). The integration vector pCM160 was constructed by cloning an 819-bp-long PCR fragment of the *citM* promoter region, *PcitM*, into the multiple cloning site of pJM116. The *citM* promoter region including the *cre* site was amplified by PCR using a forward primer (5'-CTCC AAGGAATTCGACGCGTTGCATTGCC-3') that introduced an *EcoRI* site (boldface) and a backward primer (5'-AAGCCTAAGGATCCTAACACATCC ATTCCC-3') that introduced a *BamHI* site (boldface). Both pJM116 and the *PcitM* PCR fragment were digested with *BamHI* and *EcoRI* and ligated to yield pCM160. The vector was constructed in *Escherichia coli* DH5 $\alpha$  grown in Luria-Bertani (LB) medium (30) at 37°C. Transformants were selected by including 50  $\mu$ g of ampicillin/ml in LB agar plates. The construct was checked by restriction and DNA sequence analyses.

Wild-type and mutant *B. subtilis* strains were transformed with pCM160 to yield the CM series of mutants listed in Table 1. Successful integrants into the *amyE* locus by homologous recombination were selected for by resistance against chloramphenicol. Integration into the *amyE* locus was confirmed by an amyE-negative phenotype of cells plated on LB agar containing soluble starch (16). Integrants contained the *lacZ* gene under the control of the *citM* promoter region.  $\beta$ -Galactosidase activity was assayed qualitatively on LB agar plates containing the chromogenic substrate 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal) with or without trisodium citrate (10 mM).

**Transport assay.** Cells from 20-ml cultures were resuspended in 50 mM PIPES, pH 6.5, to yield an  $OD_{660}$  of 10 and stored on ice until use. Transport activity was determined by the rapid-filtration method (27). Briefly, cells were diluted 10-fold in 50 mM PIPES, pH 6.5–1 mM  $NiCl_2$  and incubated for 5 min at 30°C. At time zero, 1  $\mu$ l of [ $^{14}C$ ]citrate (114 mCi/mmol) was added to 99  $\mu$ l of cell suspension, yielding a final concentration of 4.4  $\mu$ M citrate. Uptake was stopped by the addition of 2 ml of ice-cold 0.1 M LiCl solution, immediately followed by filtration through a 0.45- $\mu$ m-pore-size nitrocellulose filter. The filters were washed once with the same LiCl solution. The filters were submerged in scintillation fluid, and the retained radioactivity was counted in a liquid scintil-

lation counter. Samples were taken at time points between 0 and 10 min. Uptake rates were determined from the linear initial part of each uptake curve.

Speciation of  $Ni^{2+}$  in the transport assay buffer was calculated using the MINTQA2 program (21). The concentration of  $NiCl_2$  used during the transport studies was 1 mM, which drives 99.9% of the radiolabeled citrate into the complexed state.

**$\beta$ -Galactosidase assay.** One milliliter of cell culture at an  $OD_{660}$  between 0.3 and 0.6 (exponential growth phase) was harvested by centrifugation for 5 min in an Eppendorf table top centrifuge operated at 14,000 rpm. Cell extracts were obtained by lysozyme treatment, and  $\beta$ -galactosidase activities were determined using *o*-nitrophenyl- $\beta$ -D-galactopyranoside as the substrate, as described previously (30).  $\beta$ -Galactosidase activities of *PcitM-lacZ* integrants were corrected for  $\beta$ -galactosidase activity of *B. subtilis* 168 transformed with pJM116, which amounted to 0.4 to 2.5 Miller units. Strain CM004 contains the *PcitM-lacZ* fusion integrated in the chromosome of strain SA003, which already contains another *lacZ* fusion (Table 1). Under the growth conditions used in our experiments the  $\beta$ -galactosidase activity of strain SA003 was the same as that observed for strain 168 transformed with plasmid pJM116. Consequently, the  $\beta$ -galactosidase activity of strain CM004 reflects *citM* promoter activity.

#### RESULTS

**Induction and glucose catabolite repression of *citM* expression.** *B. subtilis* contains two known transporters for citrate, CitH and CitM. CitM is responsible for citrate-induced citrate uptake activity and transports citrate in complex with  $Mg^{2+}$  and other divalent metal ions (2, 3). To study the expression of CitM, the uptake activity was measured using the  $Ni^{2+}$ -citrate complex as the substrate, which is highly specific for CitM (Krom et al., submitted).  $Ni^{2+}$  was chosen rather than  $Mg^{2+}$  because of the higher stability of the  $Ni^{2+}$ -citrate complex,

TABLE 1. *B. subtilis* strains used in this study

Strain	Genotype <sup>a</sup>	Source (reference[s])
168	<i>trpC2</i>	Microbiology, University of Groningen
SA003	<i>trpC2 sacB'-lacZ ptsH1</i>	J. Deutscher (7)
QB7097	<i>trpC2 crh::spc</i>	I. Martin-Verstraete
QB7102	<i>trpC2 ptsH1 crh::aphA3</i>	I. Martin-Verstraete (14, 34)
QB5407	<i>trpC2 ccpA::Tn917 spc</i>	I. Martin-Verstraete (10)
CM002	<i>trpC2 amyE::(PcitM-lacZ cat)</i>	This study
CM004	<i>trpC2 sacB'-lacZ ptsH1 amyE::(PcitM-lacZ cat)</i>	This study
CM006	<i>trpC2 crh::spc amyE::(PcitM-lacZ cat)</i>	This study
CM008	<i>trpC2 ptsH1 crh::aphA3 amyE::(PcitM-lacZ cat)</i>	This study
CM010	<i>trpC2 ccpA::Tn917 spc amyE::(PcitM-lacZ cat)</i>	This study

<sup>a</sup> Tn917 *spc*, Tn917 derivative conferring resistance to spectinomycin; *aphA3*, *Enterococcus faecalis* kanamycin resistance gene; *cat* pC194 chloramphenicol acetyltransferase gene.

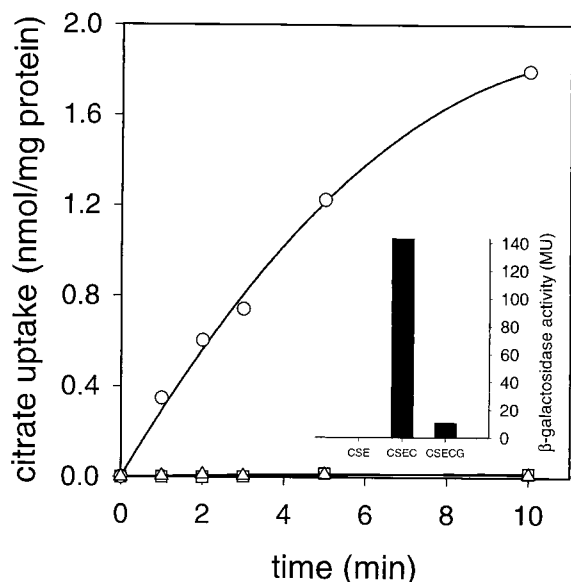


FIG. 2. Ni<sup>2+</sup>-citrate uptake and *citM* promoter activity. Uptake of [1,5-<sup>14</sup>C] citrate in whole cells of *B. subtilis* 168 grown in CSE medium without further additions (□, CSE), with 10 mM citrate (○, CSEC), and with 10 mM citrate plus 10 mM glucose (△, CSECG). (Inset) β-Galactosidase activity (in Miller units [MU]) of *B. subtilis* CM002 carrying the *lacZ* gene under the control of the *citM* promoter grown in the same media.

which assures that all citrate is in the complexed state (log  $K_A$  is 5.4 and 3.4 for Ni<sup>2+</sup> and Mg<sup>2+</sup>, respectively [28]).

*B. subtilis* 168 grown in minimal medium containing succinate and glutamate (CSE medium) showed no uptake of Ni<sup>2+</sup>-citrate, while growth in the same medium with additional citrate resulted in significant uptake (Fig. 2). When, in addition to citrate, glucose was added, the uptake activity dropped dramatically. The experiment suggests that Ni<sup>2+</sup>-citrate uptake activity is induced by citrate and repressed by glucose.

To correlate the Ni<sup>2+</sup>-citrate uptake activity with the expression of the *citM* gene, the gene encoding β-galactosidase was fused behind the promoter region of *citM* (*PcitM-lacZ* fusion) and the construct was integrated in the *amyE* locus on the genome of *B. subtilis* 168, yielding strain CM002 (Table 1). The β-galactosidase activity of CM002 correlated with the Ni<sup>2+</sup>-citrate uptake activity observed in the wild-type strain. No β-galactosidase activity was seen when cells were grown in the absence of citrate, while a high activity was observed in the presence of citrate. Including glucose in the medium in addition to citrate resulted again in very low β-galactosidase activity (Fig. 2, inset). The correlation between Ni<sup>2+</sup>-citrate uptake activity and *citM* promoter activity indicates that the lack of uptake activity in cells grown in the absence of citrate or in the presence of glucose was due to the lack of expression of *citM*.

The same pattern of induction and glucose repression was observed upon growth of *B. subtilis* in minimal medium containing inositol (CI medium) and inositol plus glutamate (CIE medium) (Table 2). In the absence of citrate neither significant uptake of Ni<sup>2+</sup>-citrate nor promoter activity was observed. In the presence of citrate both uptake and promoter activities were significantly higher in CSE medium than in CI and CIE media, while repression by glucose was most effective in the two media that contained glutamate in addition to succinate or inositol (CSE and CIE media, respectively).

**Ni<sup>2+</sup>-citrate uptake and *citM* promoter activity in different growth media.** The repressive effects of various growth media on Ni<sup>2+</sup>-citrate uptake and *citM* promoter activity were deter-

TABLE 2. Ni<sup>2+</sup>-citrate uptake activity and *PcitM* promoter activity in minimal media

Medium	Initial uptake rate (nmol/min · mg of protein)	β-Galactosidase activity (Miller units)
CI	0.01	0
CI + citrate	0.18	35
CI + citrate + glucose	0.10	10
CSE	0.01	0
CSE + citrate	0.38	143
CSE + citrate + glucose	0.01	11
CIE	0.01	0
CIE + citrate	0.17	30
CIE + citrate + glucose	0.01	2

mined by growing *B. subtilis* in citrate minimal medium supplemented with different carbon sources. Highest uptake and promoter activities were observed when cells were grown in C medium with citrate as the sole carbon and energy source (Fig. 3). Supplementing the growth medium with the carbohydrate glucose, glycerol, or inositol resulted in dramatic loss of both activities. The repressive effect was not restricted to sugars, because, though less prominent, clear decreases in both Ni<sup>2+</sup>-citrate uptake activity and *citM* promoter activity were observed with cells grown in medium containing succinate and glutamate. The similar activities of cells grown in inositol and inositol-glutamate media suggest that the latter repression is caused by succinate. In all the growth media tested, there was a fair correlation between CitM transport activity and *citM* promoter activity, with the relatively low promoter activity in the glycerol medium as the exception. The differences between the two activities most likely represent other regulatory factors acting after the transcription of the gene or differences in the metabolic state of the cells (see Discussion).

The highest growth rate was observed in the medium with the lowest Ni<sup>2+</sup>-citrate uptake activity and *citM* promoter activity. The doubling time in the media supplemented with glucose, glycerol, or inositol ranged from 120 to 140 min. The

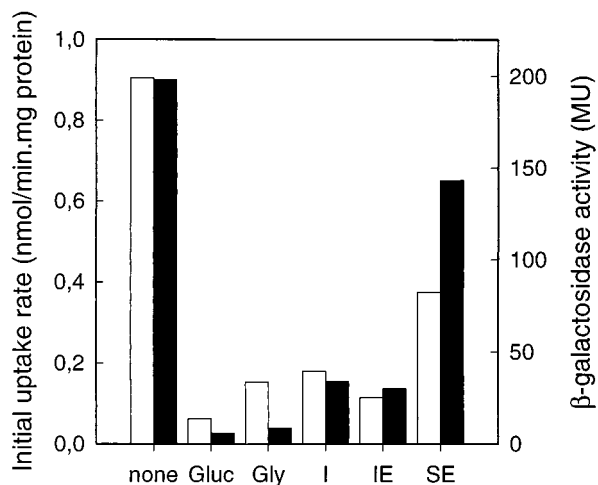


FIG. 3. Effect of different growth substrates on transport and promoter activities. *B. subtilis* strains 168 and CM002 were grown in C minimal medium with 10 mM citrate in the presence of no further additions (none), glucose (Gluc), glycerol (Gly), inositol (I), inositol and glutamate (IE), and succinate and glutamate (SE). Open bars, initial rates of uptake of [1,5-<sup>14</sup>C]citrate in the presence of 1 mM NiCl<sub>2</sub> by whole cells of *B. subtilis* 168; solid bars, β-galactosidase activity of *B. subtilis* CM002 grown in the different media in Miller units (MU).

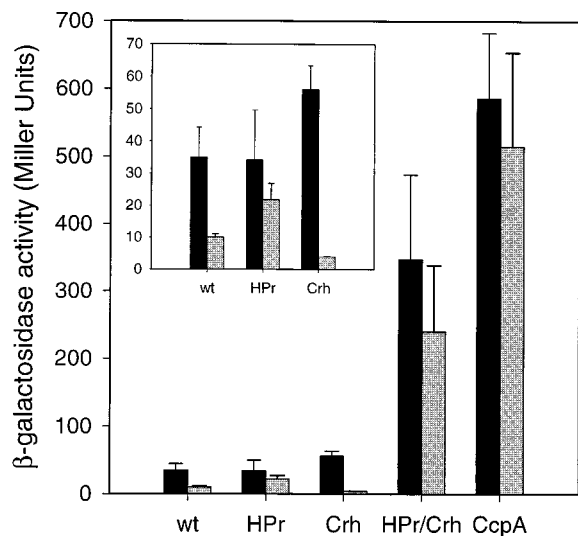


FIG. 4. *citM* promoter activity of CCR mutants grown in CI medium.  $\beta$ -Galactosidase activities of *B. subtilis* strains CM002 (wt), CM004 (HPr), CM006 (Crh), CM008 (HPr/Crh), and CM010 (CcpA) grown in CI medium supplemented with citrate (black bars) and citrate plus glucose (gray bars) are shown. All strains carry the *PcitM-lacZ* fusion integrated in the *amyE* locus. (Inset) enlarged part of the graph.

lowest growth rate was observed in minimal citrate medium (380-min doubling time), while in CSE medium growth rate and *citM* expression were intermediate (230-min doubling time).

In conclusion, the expression of the  $\text{Ni}^{2+}$ -citrate uptake system is under strict control of the components of the growth medium and is repressed by other growth substrates besides glucose. The level of expression of *CitM* was inversely related to the growth rates in the different media, which is typical for CCR.

**Roles of HPr, Crh, and CcpA in repression of *citM* expression in CI medium.** *B. subtilis* mutants SA003 (genotype *ptsHI*), QB7097 (*crh*), and QB5407 (*ccpA*) are defective in HPr, Crh, and CcpA, respectively, components involved in CCR in *B. subtilis* (see the introduction) (Table 1). Mutant QB7102 is a double mutant defective in the two homologous proteins HPr and Crh. The *PcitM-lacZ* fusion was integrated into the chromosome of each of these mutants to measure the promoter activity in the mutant background under inducing and repressing conditions.

*CitM* promoter activity in wild-type cells grown in CI medium was approximately sixfold lower than that observed in a medium with only citrate (CC medium) (Fig. 3). The activity was repressed 3.5-fold when glucose was also present in the medium (Fig. 4). Surprisingly, the *ccpA* mutant strain showed a 17-fold increase in  $\beta$ -galactosidase activity compared to the wild-type level when grown in the absence of glucose in CI medium. As expected, no significant glucose repression was observed in the mutant. The results show that both inositol and glucose have a repressive effect on *citM* expression in the wild-type strain and that the repression by both is mediated via CcpA. In agreement, a significantly elevated  $\beta$ -galactosidase activity was observed in the *ptsHI crh* double mutant, but the activity was somewhat lower than that of the *ccpA* mutant. HPr, Crh, or both are involved in inositol repression in addition to CcpA. Glucose repression was almost completely alleviated in the double mutant. In contrast to the double mutant, the *ptsHI* and *crh* single-mutant strains showed wild-type promoter activity levels when grown in CI medium. Apparently,

TABLE 3. Initial uptake rates of  $\text{Ni}^{2+}$ -citrate in wild-type and mutant *B. subtilis* strains

Medium	Initial uptake rate (nmol/min · mg of protein) for strain <sup>a</sup> :				
	168 (wild type)	SA003 ( <i>ptsHI</i> )	QB7097 ( <i>crh</i> )	QB7102 ( <i>ptsHI crh</i> )	QB5407 ( <i>ccpA</i> )
CI + citrate	0.18	0.20	0.21	2.42	2.50
CI + citrate + glucose	0.10	0.13	0.08	1.13	1.44
CSE + citrate	0.38	0.80	0.76	2.02	1.87
CSE + citrate + glucose	0.01	0.02	0	0.61	1.74
CIE + citrate	0.17	0.06	0.08	1.03	1.89
CIE + citrate + glucose	0.01	0.01	0.01	1.09	1.57

<sup>a</sup> The relevant genotype is in parentheses.

both proteins are involved in the repression of *citM* expression by inositol but they can replace one another. Compared to the wild type, repression by glucose was less strong in the *ptsHI* single mutant but was stronger in the *crh* mutant, where repression was very potent (Fig. 4, inset). This suggests an important role for HPr in glucose repression in CI medium.

Initial uptake rates of  $\text{Ni}^{2+}$ -citrate in wild-type and mutant cells correlated well with  $\beta$ -galactosidase activity under the same conditions (Table 3). The results showed similar induction and repression features. Transport activity was 1 order of magnitude higher in the *ccpA* mutant and the *ptsHI crh* double mutant than in the wild-type strain. The repression of transport activity by inositol was equally well relieved in the *ccpA* mutant and the *ptsHI crh* double mutant, but in both mutants there was still significant repression by glucose. The transport activities of the *crh* and *ptsHI* single mutants paralleled the promoter activities. Repression by inositol was not relieved in the single mutants, while glucose repression was most potent in the *crh* mutant and less so in the *ptsHI* mutant.

**Roles of HPr, Crh, and CcpA in repression of *citM* expression in CSE and CIE media.** Upon growth of cells in the CSE and CIE media, the main features of promoter activity and  $\text{Ni}^{2+}$ -citrate uptake activity of the mutant and wild-type strains were similar to those observed in CI medium (Tables 3 and 4). Importantly, in the succinate-glutamate medium (CSE medium) both transport and promoter activities were four- to fivefold higher in the *ccpA* mutant and *ptsHI crh* double mutant than in the wild-type strain, indicating that the medium components (succinate and/or glutamate) repressed *citM* expression via the CCR system. Noteworthy is that in CSE medium the double mutant and the *ccpA* mutant gave comparable

TABLE 4. *PcitM* promoter activity measured in wild-type and mutant *B. subtilis* strains

Medium	$\beta$ -Galactosidase activity (Miller units) for strain <sup>a</sup> :				
	CM002 (wild type)	CM004 ( <i>ptsHI</i> )	CM006 ( <i>crh</i> )	CM008 ( <i>ptsHI crh</i> )	CM010 ( <i>ccpA</i> )
CI + citrate	35	34	56	347	586
CI + citrate + glucose	10	22	4	240	515
CSE + citrate	143	276	460	525	525
CSE + citrate + glucose	11	22	16	344	526
CIE + citrate	30	29	30	228	430
CIE + citrate + glucose	2	6	2	304	481

<sup>a</sup> The relevant genotype is in parentheses.

$\beta$ -galactosidase and transport activities, indicating that repression by succinate and/or glutamate is completely mediated via HPr and/or Crh. In the two single mutants, both transport activity and promoter activity were stimulated by a factor of two relative to those of the wild-type strain, indicating that both HPr and Crh play a role in succinate and/or glutamate repression but that they cannot completely take over each other's roles as was observed in repression by inositol. Additional repression by glucose in the wild-type and mutant strains was much stronger in CSE medium than in CI medium, except for the *ccpA* mutant, where repression was completely relieved.

CitM expression is repressed in CSE medium. Unfortunately, *B. subtilis* did not grow on C medium containing succinate as the sole carbon and energy source and only poorly on glutamate, which did not allow discrimination between succinate and glutamate as the repressive substrate. As an alternative, all experiments were repeated using CI medium to which glutamate was added (CIE medium). The addition of glutamate had no effect on the repression by inositol (Tables 3 and 4). However, there was a marked difference in the repression by glucose in this medium. The repression was much stronger than in the absence of glutamate and comparable to the repression in CSE medium.

**Growth defects of the *ptsHI*, *crh*, and *ccpA* mutant strains.** A (partly) functional CCR system was necessary for normal growth on inositol. Both the *ptsHI crh* double mutant and the *ccpA* mutant grew poorly in CI and CIE minimal media, with growth rates of 0.07 to 0.11 h<sup>-1</sup> compared to 0.32 (CI medium) and 0.69 h<sup>-1</sup> (CIE medium) for the wild-type strain. Especially long lag phases were observed, and the addition of glucose, citrate, or glutamate (44) had no significant effect on the growth rate.

## DISCUSSION

In *B. subtilis* the uptake of citrate is mediated by at least two known homologous secondary transport proteins (3). One of them, termed CitM, is a proton motive force-driven transporter that mediates the transport of citrate complexed to Mg<sup>2+</sup> and several other divalent metal ions in symport with two protons (3). CitM is believed to be the predominant citrate transporter under physiological conditions (2, 3). The conditions under which CitM is expressed were the topic of our research. The expression of *citM* was monitored at the transcriptional level by measuring the *citM* promoter activity and at the protein level by measuring Ni<sup>2+</sup>-citrate uptake activity in whole cells. Our results indicate that *citM* expression is under strict control of the medium composition. CitM is an inducible protein that is only expressed when citrate is present in the growth medium, which is sensed by the CitS-CitT two-component system, whose coding sequence is located upstream of *citM* (9; Yamamoto et al., Abstr. 10th Int. Conf. Bacilli). The expression was highest when citrate was the only carbon and energy source in the medium. The carbohydrates glucose, glycerol, and inositol are preferred over citrate, resulting in higher growth rates and a strongly repressed expression of *citM*. Remarkably, the expression of *citM* was also repressed during growth on the nonsugars succinate and glutamate, albeit to a lesser extent. In *B. subtilis*, repression by growth substrates other than glucose has been reported for inositol dehydrogenase, which was repressed by glycerol and mannitol (7), for the *hut* operon, that was found to be repressed by amino acids (43) and for the first three enzymes of the Krebs cycle were found to be repressed by glutamate and glutamine (8, 33).

Transcription of the structural gene is the first step in the biosynthetic pathway of a protein. The amount of a membrane

protein such as CitM that ends up in the cytoplasmic membrane depends on the rate of transcription of the gene (measured by the promoter activity) and on other factors such as messenger stability, efficiency of insertion into the membrane, and protein stability. The activity of the ensemble of protein molecules in the membrane, which is the relevant parameter for the cell, may further depend on regulation of the activity of the individual protein molecules by global cellular factors such as pH and redox potential and by more-specific effectors. Most importantly, for secondary transporters, transport activity depends on the energy status of the cell. Transport activity catalyzed by CitM is driven by the electrochemical proton gradient that is maintained across the cytoplasmic membrane by the cellular energy metabolism (3). In this study, a reasonable correlation between *citM* promoter activity and Ni<sup>2+</sup>-citrate uptake activity in the different strains and under different growth conditions was observed; this is somewhat surprising considering the above discussion. Apparently, in the media tested, the uptake of citrate complexed to divalent metal ions in *B. subtilis* is mainly regulated at the level of transcription of the *citM* gene.

The promoter region of *citM* contains a *cre* sequence centered 24 bp upstream of the *citM* start codon. The 14-bp DNA sequence with dyad symmetry deviates very little from the consensus sequence (41, 46) (Fig. 1), which suggested that CitM is likely to be subject to carbon catabolite repression. It has been suggested that A- and T-rich regions intensify the interactions of catabolite control protein A (CcpA) with the *cre* site flanking the *citM cre* sequence (46). Recently, it was demonstrated that the *citM cre* site is active in vivo (32). The strong relief of *citM* repression in all media tested in a defective-CcpA mutant indicates that repression by not only glucose but also inositol and succinate and/or glutamate in the wild-type strain is mediated by the binding of CcpA to the *cre* site located in the *citM* promoter region.

In CCR, the binding of CcpA to the *cre* site is induced by complex formation of CcpA and the phosphorylated forms of HPr and Crh. Repression of *citM* expression in CI medium was less relieved in the *ptsHI crh* double mutant than in the *ccpA* mutant. Since in the double mutant the CcpA molecule is present, this may indicate an affinity of uncomplexed CcpA for the *cre* site or promotion of binding by factors other than HPr and Crh (15, 23). Complete relief of repression in the double mutant when grown in CSE medium suggests that in CI medium other metabolic intermediates promote CcpA binding to the *cre* site.

The specific roles of HPr and Crh in CCR are not clear. HPr is one of the general proteins in the phosphoenolpyruvate-dependent PTS that transports sugars into the cell with concomitant phosphorylation. HPr is a phosphocarrier intermediate that is phosphorylated at a histidine residue (His15) by phosphoenolpyruvate in a reaction catalyzed by enzyme I (EI). It donates its phosphoryl group to a sugar-specific enzyme or enzyme domain termed IIA. In CCR, HPr is phosphorylated by ATP at a serine residue (Ser46), a reaction catalyzed by HPr kinase. It has been suggested that the functions of HPr in the PTS and CCR interact when transcription of a gene is repressed by a PTS sugar, for instance, glucose (35). The Crh protein is not operational in the PTS (29), simply because, at the position corresponding to the phosphorylation site (His15) in HPr, a glutamine residue is found in Crh. The CCR phosphorylation site (Ser46) is present, and it has been shown that Crh is phosphorylated by HPr kinase in vitro (13, 14). In a number of studies, it has been observed that HPr and Crh can take over each other's role in the CCR function (34, 46). Similarly, in this study, repression of expression of *citM* by

inositol in the *ptsHI* and *crh* single mutants was similar to that observed in the wild-type strain, while repression was alleviated in the *ptsHI crh* double mutant (Fig. 4). It should be noted that inositol is believed to be taken up by the cell by a secondary transporter (encoded by *iolF*) (45) and, therefore, that there is no turnover of the PTS during growth on inositol. For a PTS sugar, it may be anticipated that the CCR system discriminates between HPr and Crh (35). In agreement, repression of *citM* by glucose is more effective in the *crh* mutant than in the HPr mutant, suggesting that repression by HPr is potentiated during turnover of the PTS. Discrimination between HPr and Crh in the repression of *citM* in medium containing the nonsugars succinate and glutamate (CSE medium) was also observed in this study. Repression was relieved twofold in both single mutants, suggesting that HPr could only partly take over repression exerted by Crh and vice versa. The mechanism by which succinate and/or glutamate metabolism affects the degree of phosphorylation of HPr and Crh is completely unknown.

The addition of glucose to CI medium resulted in an additional threefold repression of *citM* expression. The addition of glutamate to CI medium had no effect on *citM* expression. Remarkably, when both glucose and glutamate were added to CI medium, the additional repression was about 15-fold and of the same order of magnitude of the glucose repression in CSE medium, which also contains glutamate. This suggests that the presence of glutamate in the medium makes repression by glucose much stronger. A similar synergistic repression has been observed for the *citB* gene of *B. subtilis*, coding for the tricarboxylic acid (TCA) cycle enzyme aconitase. Expression of the *citB* gene in minimal medium containing glucose or glycerol was fully repressed when a source of 2-ketoglutarate, such as glutamine or glutamate, was present (33, 37, 39). Repression of *citB* is mediated by a novel regulator termed CcpC (22). A *PcitM-lacZ* fusion integrated in the *amyE* locus of *B. subtilis* CJB8 containing an inactivated *ccpC* gene resulted in the same  $\beta$ -galactosidase activities when grown on CSE medium in the presence and absence of glucose as those observed for the wild-type strain CM002 (not shown). This strongly suggests that CcpC is not involved in the regulation of *CitM* expression and that synergistic repression is not restricted to regulation by CcpC.

The *ccpA* mutant has been reported to be unable to grow on glucose minimal medium with ammonium as the nitrogen source (44). The observation was explained by the inability of the mutant to utilize ammonium as a single source of nitrogen because of the absence of the key enzyme of ammonium assimilation, glutamate synthase (10). Addition of TCA cycle intermediates, such as citrate and glutamate, could restore growth on minimal medium containing glucose and ammonium (44), but not on that containing arabinose (38). In this study, the effect of inositol was as observed with arabinose, i.e., growth in CI and CIE medium was severely slowed down even when citrate or glutamate or both were present. The same was true for the *ptsHI crh* double mutant grown in minimal medium in the presence of inositol, whereas the *ptsHI* and *crh* single mutants showed wild-type growth rates under these conditions. Similar growth defects have been described by Zalićkas et al. (47).

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#### ADDENDUM IN PROOF

An extensive characterization of the *citM* promoter region was recently published (H. Yamamoto, M. Murata, and J. Sekiguchi, *Mol. Microbiol.* **37**:898–912, 2000).

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