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CcpA-Independent Regulation of Expression of the Mg^{2+}-Citrate Transporter Gene *citM* by Arginine Metabolism in *Bacillus subtilis*

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Transcriptional regulation of the Mg^{2+}-citrate transporter, CitM, the main citrate uptake system of *Bacillus subtilis*, was studied during growth in rich medium. Citrate in the growth medium was required for induction under all growth conditions. In Luria-Bertani medium containing citrate, *citM* expression was completely repressed during the exponential growth phase, marginally expressed in the transition phase, and highly expressed in the stationary growth phase. The repression was relieved when the cells were grown in spent Luria-Bertani medium. The addition of a mixture of 18 amino acids restored repression. L-Arginine in the mixture appeared to be solely responsible for the repression, and ornithine appeared to be an equally potent repressor of *citM* expression. Studies of mutant strains deficient in RocR and SigL, proteins required for the expression of the enzymes of the arginase pathway, confirmed that uptake into the cell and, most likely, conversion of arginine to ornithine were required for repression. Arginine-mediated repression was independent of a functional CcpA, the global regulator protein in carbon catabolite repression (CCR). Nevertheless, CCR-mediated repression was the major mechanism controlling the expression during exponential growth, while the newly described, CcpA-independent arginine-mediated repression was specifically apparent during the transition phase of growth.

The Mg^{2+}-citrate transport protein, CitM, is believed to be the predominant citrate uptake system in *Bacillus subtilis* under aerobic growth conditions (30). Its ability to transport citrate is dependent on the presence of a well-defined set of divalent metal ions, Mg^{2+}, Mn^{2+}, Zn^{2+}, Ni^{2+}, and Co^{2+}. The divalent metal ions are transported in complex with citrate into the cell (15). More recently, it was demonstrated that D-isocitrate, like citrate, is transported by CitM when complexed to the same set of divalent metal ions (30). The metal ion specificity of the complex renders *B. subtilis* extremely sensitive to the toxic ions Zn^{2+}, Ni^{2+}, and Co^{2+} in the presence of citrate or isocitrate when CitM is present in the membrane (14). Uptake of the metal citrate complex catalyzed by CitM is driven by the electrochemical gradient of protons that is maintained across the cell membrane (proton motive force). Translocation was shown to be electrogenic, i.e., CitM couples the uptake of the monovalent Me^{2+}:$citrate^{3-}$ complex to the uptake of at least two protons (3).

Expression of the Mg^{2+}-citrate transporter of *Bacillus subtilis* has been studied extensively and was shown to be regulated at the transcriptional level. In minimal medium, expression of the *citM* gene requires the presence of citrate or isocitrate in the medium. Induction is mediated by the two-component system CitST (34), of which the sensor kinase CitS recognizes external citrate or isocitrate and the response regulator CitT works as a transcription activator, by binding to the promoter region of *citM*. Furthermore, *citM* expression is repressed by more-favorable carbon sources like glucose via the carbon catabolite repression (CCR) system. In addition to that by glucose, CCR-mediated repression of *citM* expression by glycerol and inositol and by a combination of the nonsugars succinate and glutamate (29) has been reported. Major players in CCR in gram-positive organisms are the global regulator CcpA and its coeffectors HPr and Crh (reviewed in reference 27). CcpA is a member of the LacI-GalR family of regulatory proteins (12) that binds to its cognate operator site, the so-called *cre* (catabolite-responsive element) site (13), where it acts as a repressor or activator of transcription (28). *cre* sites are located within or near the promoter region of the targeted genes (20). Binding of CcpA to these palindromic DNA sequences is driven by complex formation with the Ser46-phosphorylated forms of HPr, the general phosphocarrier protein of the phosphoenolpyruvate-dependent sugar uptake system (phosphotransferase system), or Crh, a protein homologous to HPr (8, 16), which, however, has no function in the phosphotransferase system (9, 17). Mutants deficient in CcpA, HPr, and/or Crh have been shown to result in (partial) relief of repression or activation of several genes (6, 9, 27, 28), including the *citM* gene (29).

In this study, the *citM* expression pattern during growth in rich (Luria-Bertani [LB]) medium was investigated. It is shown that *citM* expression follows a complex pattern in which CCR plays a major role during the exponential growth phase, after which a new repression mechanism involving arginine metabolism takes over in the transition phase of growth. It is dem-

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

Strain	Genotype[a]	Source or reference
168	*trpC2*	This laboratory
CM001	*trpC2 amyE*::(*lacZ cat*)	29
CM002	*trpC2 amyE*::(P*citM-lacZ cat*)	29
CM008	*trpC2 ptsH1 crh*::*aphA3* *amyE*::(P*citM-lacZ cat*)	29
CM010	*trpC2 ccpA*::Tn*917 spc* *amyE*::(P*citM-lacZ cat*)	29
QB5407	*trpC2 ccpA*::*spc*	21
QB5533	*trpC2 rocR*::*aphA3*	4
QB5505	*trpC2 sigL*::*aphA3*	5
QB5619	*trpC2 ahrC*::*aphA3* *amyE*::*rocD'-'lacZ*	11
CM021	*trpC2 rocR*::*aphA3* *amyE*::(P*citM-lacZ cat*)	This study
CM023	*trpC2 sigL*::*aphA3* *amyE*::(P*citM-lacZ cat*)	This study
CM031	*trpC2 ccpA*::Tn*917 spc* *rocR*::*aphA3* *amyE*::(P*citM-lacZ cat*)	This study
SF62	*trpC2 tnrA62*::Tn*917 erm*	33
HJ31	*trpC2* Δ*glnR*	A. L. Sonenshein
PS252	*trpC2 codY*::*tet*	A. L. Sonenshein
CM040	*trpC2 abrB*::*spc*	This laboratory
CM041	*trpC2 spo0A*::*erm*	This laboratory
CM043	*trpC2 tnrA62*::Tn*917 erm* *amyE*::(P*citM-lacZ cat*)	This study
CM044	*trpC2* Δ*glnR* *amyE*::(P*citM-lacZ cat*)	This study
CM045	*trpC2 codY*::*tet* *amyE*::(P*citM-lacZ cat*)	This study
CM046	*trpC2 abrB*::*spc* *amyE*::(P*citM-lacZ cat*)	This study
CM047	*trpC2 spo0A*::*erm* *amyE*::(P*citM-lacZ cat*)	This study

[a] Tn*917 spc*, Tn*917* derivative conferring resistance to spectinomycin; *aphA3, Enterococcus faecalis* kanamycin resistance gene; *cat*, pC194 chloramphenicol acetyltransferase gene.

onstrated that CcpA, the global regulator of CCR, was not involved in the newly described arginine-potentiated repression mechanism.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. subtilis* 168-derived strains used in this study are listed in Table 1. Strain CM002 containing the P*citM-lacZ* promoter fusion is referred to in this study as the wild type. Precultures of *B. subtilis* wild-type and mutant strains were grown overnight at 37°C in LB medium. The precultures were diluted 100 times in fresh or spent LB medium supplemented with 10 mM trisodium citrate (LBC and spent LBC, respectively). In addition, the auxotrophic requirement tryptophan was added to spent LB medium. Spent LB medium was prepared by growing the wild-type strain for 6.5 h in LB medium, after which cells were removed by centrifugation for 5 min at 10,000 × *g* and at 4°C. The supernatant was centrifuged once again under the same conditions and, subsequently, filter sterilized and stored at 4°C until use. Since, quantitatively, the results with the different batches of spent medium were variable, batch numbers are given in the Results section.

The final concentrations of the amino acids in a mixture of 18 amino acids (M18 mixture) that was added to spent LB medium were the same as indicated by the manufacturer in fresh LB: L-alanine, 6.2 mM; L-arginine, 2.6 mM; L-aspartate, 7.1 mM; L-cystine, 0.3 mM; L-glutamate, 16.4 mM; glycine, 4.5 mM; L-histidine, 1.7 mM; L-isoleucine, 4.6 mM; L-leucine, 7.2 mM; L-lysine, 6.3 mM; L-methionine, 2.1 mM; L-phenylalanine, 3.0 mM; L-proline, 7.6 mM; L-serine, 5.4 mM; L-threonine, 4.2 mM; L-tryptophan, 0.7 mM; L-tyrosine, 1.1 mM; and L-valine, 5.9 mM. The M5 mixture contained L-arginine, L-aspartate, L-glutamate, L-histidine, and L-proline at the same concentrations. Other growth substrates were added at the same concentration as L-arginine, 2.6 mM.

All cells were grown in flasks at 37°C under continuous shaking at 150 rpm. Growth was monitored by measuring the optical density at 660 nm (OD_{660}). When appropriate, the following antibiotics were added at the indicated concentrations: chloramphenicol, 5 μg/ml; kanamycin, 5 μg/ml; spectinomycin, 100 μg/ml; erythromycin, 1 μg/ml; and tetracycline, 10 μg/ml.

Construction of mutant strains. Mutants CM021 and CM023 were constructed by transformation of chromosomal DNA extracted from the *rocR* and *sigL* mutant strains, respectively, to strain CM002, containing the P*citM-lacZ* promoter fusion in the *amyE* site (29). Successful recombinations were selected for by resistance against chloramphenicol and kanamycin. The double mutant CM031 was constructed by transformation of chromosomal DNA extracted from the *rocR* mutant strain to strain CM010, a CcpA-deficient strain, and was selected for by resistance against the same two antibiotics plus spectinomycin.

β-Galactosidase assay. β-Galactosidase activity was determined at 28°C by the method of Miller using *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrate (19). Cells from 2 ml of culture were harvested by centrifugation, frozen, and stored at −20°C. The cell pellet was resuspended in a buffer containing 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, and 1 mM 1,4-dithiothreitol, pH 7.0, and the cells were lysed by treatment with lysozyme in the presence of 10 μM DNase. The assay was started by the addition of 0.1 ml of ONPG (4.5 mg/ml) to the cell extract and stopped by the addition of 0.15 ml of 1.2 M Na_2CO_3. After a brief spin to remove cell debris, the absorption of the sample was measured at 420 nm. Specific β-galactosidase activities were expressed as the *o*-nitrophenol released per minute per cell density at 28°C (Miller units). The values reported are averages of at least two independent measurements. Background activities were measured in *B. subtilis* strain 168 containing plasmid pJM116 integrated in the chromosome (CM001) and amounted to 0.3 to 0.9 Miller unit.

RESULTS

Expression of the *citM* gene in rich medium. *B. subtilis* strain CM002 contains the gene encoding β-galactosidase fused behind the promoter region of the *citM* gene that codes for an Mg^{2+}-citrate transporter (P*citM-lacZ* fusion) integrated in the chromosome (Table 1). Growth of the strain in LB medium in the presence or absence of 10 mM citrate revealed the same growth characteristics (Fig. 1A). For the following discussion, three phases will be distinguished in the growth curves: (i) the exponential growth phase (approximately between 1 and 3 h), (ii) the transition phase (between 3 and 6 h), and (iii) the stationary growth phase (following 6 h). Clearly, in the absence of citrate no *citM* promoter activity could be detected in any of the growth phases, as evidenced by the lack of β-galactosidase activity of the cells (Fig. 1A). This is in agreement with earlier observations obtained with minimal media that showed that citrate is needed for induction of expression of the gene (29, 32, 34). Surprisingly, in the presence of citrate, no promoter activity was observed in the exponential growth phase as well. Only when the cells entered the transition phase was a low level of activity observed, which subsequently increased further in the stationary phase (Fig. 1A).

One explanation for the observed expression pattern in the presence of citrate might be that some component(s) in the medium represses *citM* expression but is gradually depleted during growth, thereby relieving the repression. To explore this possibility, spent medium was prepared by growing *B. subtilis* for 6.5 h in LB without citrate followed by removal of the cells (see Materials and Methods for details). Strain CM002 grew exponentially for about 5 to 6 h in spent LB medium prepared in this way and supplemented with 10 mM citrate (spent LBC). The growth rate was about twofold lower than that in fresh medium (Fig. 1A). More importantly, expression of *citM* occurred from the beginning of exponential growth, suggesting

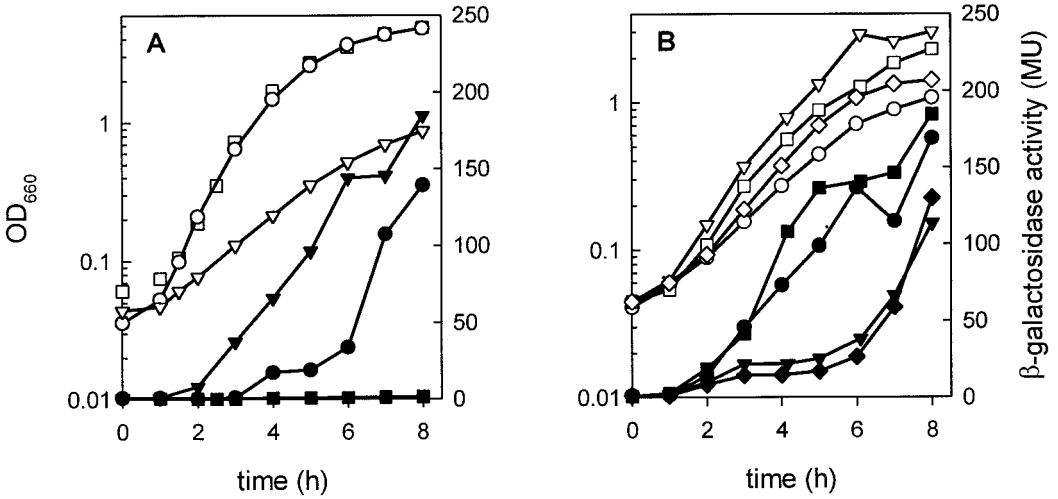

FIG. 1. Expression of *citM* in *B. subtilis* CM002 grown in fresh and spent LBC medium. β-Galactosidase activity was determined in cells grown in LB medium (■ and □), LB medium plus 10 mM citrate (● and ○), and spent LB medium plus 10 mM citrate (▼ and ▽) (A) and in spent LBC without further additions (● and ○) and supplemented with an M18 mixture (▼ and ▽), with the same mixture without L-arginine (■ and □), and with 2.6 mM L-arginine (◆ and ◇) (B). Samples were taken at the indicated time points to monitor β-galactosidase activity in Miller units (MU) (solid symbols) and growth (open symbols), measured as OD_{660}.

that the repression observed in fresh medium was relieved in spent medium (Fig. 1A).

Repression of *citM* expression by L-arginine. LB medium is known for its nitrogen-source-rich composition and primarily contains amino acids and vitamins. A mixture of 18 amino acids (M18 mixture) was added to spent medium at the same concentration as that present in fresh LB medium. The β-galactosidase activity of the cells harvested after 5 h of growth was reduced sixfold compared to that of cells grown in spent LBC alone (Table 2), suggesting repression by one or more of the amino acids. To narrow down the search for the repressor(s), a mixture of five amino acids (M5 mixture) that are metabolically linked to the tricarboxylic acid cycle in *B. subtilis* (L-glutamate, L-proline, L-arginine, L-histidine, and L-aspartate) was added to spent LBC. The mixture was equally potent in repressing the *citM* promoter activity. Subsequently, a separate addition of the five amino acids to spent medium showed that only L-arginine resulted in reduced levels of expression, while the others did not. Leaving out L-arginine from the M18 and M5 mixtures resulted in specific β-galactosidase activities similar to those observed after growth in spent LBC alone, indicating that arginine was solely responsible for the repression in the M18 and M5 mixtures.

TABLE 2. β-Galactosidase activity in *B. subtilis* CM002 grown in spent LBC medium supplemented with amino acids

Addition[a]	Batch[b]	β-Galactosidase activity (Miller units)[c]
None	I	153
None	II	99
M18 mixture	I	25
M5 mixture	I	22
L-Arginine	II	17
L-Aspartate	II	116
L-Glutamate	II	99
L-Histidine	II	140
L-Proline	II	112
M5 mixture–L-arginine	I	108
M18 mixture–L-arginine	I	136

[a] Amino acids were added at concentrations as present in fresh LB medium (see Materials and Methods).
[b] Two batches of spent LB that differed slightly in their properties were used.
[c] Activity was measured at $t = 5$ h.

The steady-state level of expression of a gene product in growing cells depends both on the promoter activity and the growth rate (31). A fair comparison of expression levels measured at a single time point in terms of promoter activities, as done above, is valid only if the growth rate under the different growth conditions is similar. Figure 1B shows that the addition of various amino acids increased the growth rates by at most 30%, not enough to account for the observed decrease in β-galactosidase activity brought about by L-arginine. The complete expression pattern during growth on spent LBC supplemented with L-arginine showed characteristics similar to those seen during growth on fresh LBC. Expression was low during the initial stages of growth, to increase drastically in the late exponential phase (compare Fig. 1A and B). Again, the difference in expression patterns during growth in the M18 mixture and the same mixture without L-arginine revealed that in the mixture L-arginine was fully responsible for the repression.

Repression of *citM* gene expression requires arginine metabolism. In *B. subtilis*, the genes located in the *roc* operons *rocABC*, *rocDEF*, and *rocG* are involved in arginine metabolism (1, 4, 10, 11). Deficiencies in RocR, a transcriptional activator needed for expression of the *roc* genes, and SigL, a σ^{54} factor required for the transcription of the arginine catabolic operons, render *B. subtilis* unable to utilize arginine (4, 5). The role of metabolism in the repression of *citM* gene expression by arginine in spent LBC medium was investigated by introducing the P*citM-lacZ* promoter fusion in strains deficient in RocR and SigL, yielding strains CM021 and CM023, respectively (Table 1). The strains grew with similar growth rates in fresh and spent LBC (data not shown).

TABLE 3. β-Galactosidase activity in *B. subtilis* CM002 grown in spent LBC medium supplemented with various nitrogen sources or arginine degradation products

Addition[a]	Batch	β-Galactosidase activity (Miller units)[b]		
		CM002 (wild type)	CM021 (*rocR*::*aphA3*)	CM023 (*sigL*::*aphA3*)
None	III	101	ND[c]	ND
None	IV	131	142	108
L-Arginine	III	37	ND	ND
L-Arginine	IV	46	106	80
Citrulline	III	91	ND	ND
Ornithine	III	41	145	111
Urea	III	103	ND	ND
Ammonium	III	80	ND	ND
α-Ketoglutarate	IV	122	ND	ND
Agmatine	IV	98	ND	ND
Putrescine	IV	128	ND	ND

[a] Nitrogen sources were added at concentrations of 2.6 mM.
[b] Activity was measured at $t = 5$ h.
[c] ND, not determined.

Strains CM021 and CM023, deficient in RocR and SigL, respectively, were grown in spent LBC in the presence and absence of L-arginine. Both the *rocR* and *sigL* mutants brought about elevated *citM* gene expression in the presence of L-arginine relative to the wild-type strain (Table 3), indicating that for L-arginine to repress *citM* expression, uptake and/or metabolism seemed to be essential. Table 3 further analyzes intermediates of arginine degradation pathways and some related products for their ability to repress *citM* expression in spent LBC. Clearly, the addition of citrulline, agmatine, putrescine, urea, α-ketoglutarate, or ammonia was without effect. However, ornithine resulted in a repression similar to that seen for arginine, suggesting that the arginine breakdown route leading to ornithine is involved in the repression. Similar to the repression by L-arginine, the repression by ornithine was lifted in the RocR and SigL mutant strains, indicating that repression by ornithine requires uptake and/or metabolism.

Arginine metabolism-linked repression in the transition phase of growth. The lack of repression by arginine and ornithine in the RocR mutant allowed for the evaluation of *citM* expression during growth in fresh LBC in the absence of arginine repression (Fig. 2). The results indicated that arginine repression was exerted specifically during the transition phase of growth. In the exponential growth phase no expression of *citM* was observed in either the wild-type strain or the RocR mutant strain. In the transition phase, the RocR-deficient strain showed rapidly increasing levels of expression, while the levels in the wild type remained low.

Involvement of CCR in *citM* expression in rich medium. *citM* gene expression is subject to CCR during growth in minimal medium. Repression was relieved in mutant strain CM010, which is deficient in CcpA, a global regulator in CCR (see Table 1). Strain CM010 grown in LBC medium showed significant β-galactosidase activity during the exponential growth phase (between 1 and 3 h), whereas the wild-type strain CM002 showed no activity (compare Fig. 1 and 3), suggesting a role for the CCR system in repression during this growth phase. Interestingly, no change in expression level was observed during the transition phase, suggesting no role of the

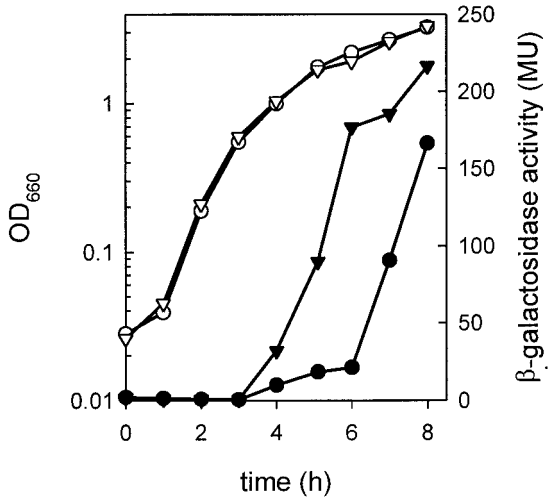

FIG. 2. Expression of *citM* in RocR-deficient cells grown in LBC medium. Strains CM002 (● and ○) and CM021 (▼ and ▽) were grown in LB medium containing 10 mM citrate. Samples were withdrawn at the indicated time points to monitor β-galactosidase activity in Miller units (MU) (solid symbols) and growth (open symbols), measured as the OD_{660}.

CCR system in arginine repression. To exclude the possibility that the expression in the exponential growth phase of the CcpA mutant would be an unspecific effect of the construction of the strain itself, not related to CCR, expression was measured under the same conditions in strain CM008, which contains the P*citM-lacZ* promoter fusion in a *ptsH1 crh* double mutant, lacking functional HPr and Crh, which prevents repression by CCR at a different level (27). The results were similar to those observed for the CcpA mutant (data not shown). In conclusion, *citM* expression in the wild-type strain is repressed during exponential growth in LBC medium, a re-

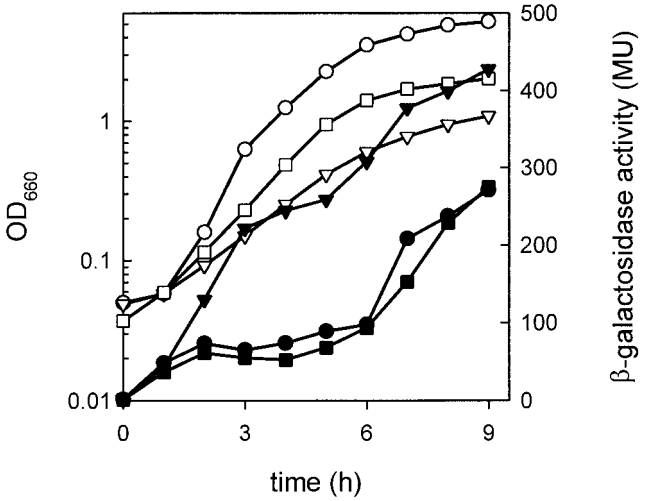

FIG. 3. Expression of *citM* in CcpA-deficient cells grown in fresh and spent LBC medium. Strain CM010 was grown in fresh LBC medium (● and ○), spent LBC medium (▼ and ▽), and spent LBC medium plus 2.6 mM L-arginine (■ and □). Samples were taken at the indicated time points to monitor β-galactosidase activity in Miller units (MU) (solid symbols) and growth (open symbols), measured as OD_{660}.

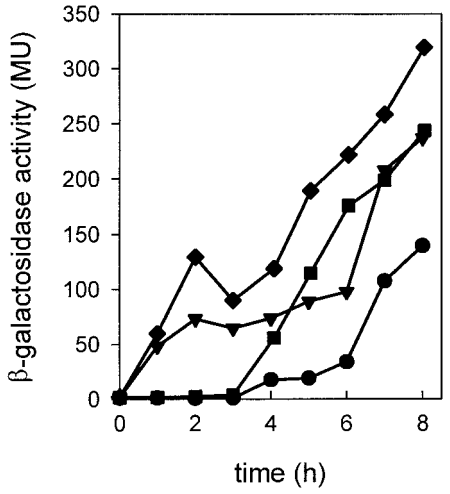

FIG. 4. Overview of expression of *citM* in repression-deficient mutants during growth in LBC medium. β-Galactosidase activity of strain CM002 (wild type) (●), CM010 (CcpA deficient) (▼), CM021 (RocR deficient) (■), and CM031 (CcpA/RocR deficient) (♦) grown in fresh LB plus 10 mM citrate. MU, Miller units.

pression that is CcpA and HPr/Crh mediated, i.e., CCR mediated.

More direct evidence for the lack of involvement of CcpA in arginine repression was obtained by growing the CM010 strain in spent LBC in the presence and absence of L-arginine. In the absence of L-arginine, the expression pattern was similar to that observed for the wild-type strain CM002 but the expression levels were approximately twofold higher over the whole growth curve (compare Fig. 1 and 3). In the presence of L-arginine, repression was exerted to the same level as that observed in fresh LBC medium, showing that arginine-mediated repression is still functional in the CcpA mutant.

A mutant strain, CM031, was constructed in which both CcpA and RocR were inactivated. Figure 4 compares the expression patterns during growth in LBC medium of the *ccpA rocR* double mutant, the RocR-deficient mutant, the CcpA-deficient mutant, and the wild-type strain CM002. It follows that the CCR-mediated repression and L-arginine repression are more or less additive processes (see also Discussion).

Several other global regulatory proteins involved in gene expression are known in *B. subtilis*, including CodY, GlnR, and TnrA, which play a role in nitrogen regulation (2, 23, 24, 33), and AbrB and Spo0A, two transcription factors involved in regulation of gene expression in the transition into the stationary growth phase (25, 26). The P*citM-lacZ* promoter fusion was introduced in strains deficient in these proteins, and the resulting strains were assayed for involvement in the repression of the *citM* gene by L-arginine as described for the RocR-deficient strain (Fig. 2). None of the regulators could be shown to be involved in the L-arginine-mediated repression (data not shown).

DISCUSSION

Expression of the Mg^{2+}-citrate transporter, CitM, of *B. subtilis* during growth in minimal media is induced by the presence of citrate or isocitrate in the medium and repressed by preferable carbon sources like glucose, glycerol, and inositol but also by the combination of the nonsugars succinate and glutamate (29). Repression is at the level of transcription and mediated by the CCR system, involving the global regulatory protein CcpA. In this study, expression of *citM* was studied in rich medium from which a complex regulation emerges. As in minimal medium, expression requires induction by citrate, but in addition, two different mechanisms of repression seem to be operative: CCR and a newly described, CcpA-independent repression involving arginine metabolism that is manifested in the transition phase of growth. The regulation of transcription of the *citM* gene during the exponential, transition, and stationary growth phases in LBC medium is summarized in Fig. 4. The *B. subtilis* wild-type strain showed no expression in the exponential growth phase, a low level of expression in the transition phase, and rapidly increasing expression in the stationary phase. Experiments with a *ccpA*-null mutant conferred elevated expression in the exponential growth phase, indicating that repression in the wild-type was CCR mediated. A *rocR*-null mutant, deficient in the enzymes of the arginine metabolic pathway, showed elevated levels of expression specifically in the transition phase of growth. Experiments using spent medium revealed that the wild-type strain was repressed by the presence of L-arginine in the medium, a repression that required the arginine metabolic enzymes. In the exponential growth phase in LBC medium, repression was not relieved in the RocR-deficient strain, indicating that CCR-mediated repression was still present. Either arginine-mediated repression is not operative in the exponential growth phase or it is overruled by CCR-mediated repression. Expression in the transition phase was not enhanced relative to the exponential growth phase in the CcpA mutant, indicating that expression was still repressed by L-arginine, consistent with the lack of relief of repression by L-arginine in the *ccpA* mutant strain grown in spent LBC. A double mutant deficient in both CcpA and RocR showed the highest levels of expression throughout the three growth phases, suggesting that the two repression mechanisms are independent. Expression of *citM* increased significantly in the stationary growth phase in all strains tested, most likely as a result of the decreased growth rate in this growth phase (31).

Transcription of *citM* is down-regulated by the global regulatory protein CcpA in response to the availability of glucose, glycerol, inositol, and succinate and/or glutamate in the medium (29). The main constituents of LB medium are amino acids, which were shown not to result in repression or CcpA-dependent repression (L-arginine) when added to spent LBC. According to the composition supplied by the manufacturer (Difco manual, 11th ed.), LB broth contains a low concentration of inositol (117 μM). The addition of inositol at the same concentration to spent LBC medium did not repress *citM* promoter activity (data not shown). No further attempts were made to identify the effector(s) of CcpA-mediated repression during the exponential growth phase. Possibly, the observed repression is the result of a mixture of effectors, all present at low concentrations.

The major pathway for the degradation of L-arginine in *B. subtilis* is the arginase pathway. The genes involved are organized in the *roc* regulon containing the *rocABC*, *rocDEF*, and *rocG* operons (1, 10, 11). Expression of the regulon requires

the presence of ornithine (11) and is mediated by the positive regulatory protein RocR, a member of the NtrC/NifA family of regulators. RocR acts together with a σ^{54} factor, SigL (4, 5). *citM* promoter activity was reduced by the presence of both arginine and ornithine in the growth medium (Tables 2 and 3). Since both *rocR*- and *sigL*-null mutants were able to overcome the repression of *citM* transcription by arginine and ornithine, it seems fair to conclude that the arginase pathway is involved in the regulation. It is likely that both arginine and ornithine have to be transported into the cell and that arginine has to be converted into ornithine to exert repression, which would imply that ornithine is the true repressor as it is the true activator for *roc* regulon activation (11).

The *citM* and *citST* (the two-component system responsible for induction of CitM) promoter regions do not contain the known σ^{54} consensus sequence (18) or RocR binding site (4), making a putative link between *roc* regulon activation and *citM* gene deactivation indirect. Several other regulatory proteins in *B. subtilis* are known to be involved in gene expression in response to amino acid availability. CodY-dependent repression occurs in cells growing rapidly in media containing amino acids (7), and GlnR and TnrA are involved during growth on amino acids, like glutamine, when used as a nitrogen source (22, 33). None of these regulators appeared to be involved in the arginine-mediated repression of *citM*. Since the repression seemed to be important, especially in the transition phase of growth, the involvement of the regulatory proteins AbrB and Spo0A, which are known to operate postexponentially (25, 26), was investigated, but these were also not involved in the repression.

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