



Malate-mediated carbon catabolite repression in bacillus subtilis involves the HPrK/CcpA pathway

Frederik M. Meyer, Matthieu Jules, Felix M. P. Mehne, Dominique Le Coq, Jens J. Landmann, Boris Goerke, Stephane Aymerich, Joerg Stuelke

► To cite this version:

Frederik M. Meyer, Matthieu Jules, Felix M. P. Mehne, Dominique Le Coq, Jens J. Landmann, et al.. Malate-mediated carbon catabolite repression in bacillus subtilis involves the HPrK/CcpA pathway. The Journal of applied bacteriology, 2011, 193 (24), pp.6939 - 6949. 10.1128/JB.06197-11 . hal-01019483

HAL Id: hal-01019483

<https://hal.science/hal-01019483>

Submitted on 29 May 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Malate-Mediated Carbon Catabolite Repression in *Bacillus subtilis* Involves the HPrK/CcpA Pathway[∇]§

Frederik M. Meyer,¹# Matthieu Jules,^{2,3}# Felix M. P. Mehne,¹# Dominique Le Coq,^{2,3,4}#
Jens J. Landmann,¹ Boris Görke,¹ Stéphane Aymerich,^{2,3*} and Jörg Stülke^{1*}

Department of General Microbiology, Institute of Microbiology and Genetics, Georg-August University Göttingen, Grisebachstr. 8, D-37077 Göttingen, Germany¹; INRA, UMRI1319 Micalis, F-78350 Jouy-en-Josas, France²; AgroParisTech, UMR Micalis, F-78350 Jouy-en-Josas, France³; and CNRS, F-78350 Jouy-en-Josas, France⁴

Received 15 September 2011/Accepted 5 October 2011

Most organisms can choose their preferred carbon source from a mixture of nutrients. This process is called carbon catabolite repression. The Gram-positive bacterium *Bacillus subtilis* uses glucose as the preferred source of carbon and energy. Glucose-mediated catabolite repression is caused by binding of the CcpA transcription factor to the promoter regions of catabolic operons. CcpA binds DNA upon interaction with its cofactors HPr(Ser-P) and Crh(Ser-P). The formation of the cofactors is catalyzed by the metabolite-activated HPr kinase/phosphorylase. Recently, it has been shown that malate is a second preferred carbon source for *B. subtilis* that also causes catabolite repression. In this work, we addressed the mechanism by which malate causes catabolite repression. Genetic analyses revealed that malate-dependent catabolite repression requires CcpA and its cofactors. Moreover, we demonstrate that HPr(Ser-P) is present in malate-grown cells and that CcpA and HPr interact *in vivo* in the presence of glucose or malate but not in the absence of a repressing carbon source. The formation of the cofactor HPr(Ser-P) could be attributed to the concentrations of ATP and fructose 1,6-bisphosphate in cells growing with malate. Both metabolites are available at concentrations that are sufficient to stimulate HPr kinase activity. The adaptation of cells to environmental changes requires dynamic metabolic and regulatory adjustments. The repression strength of target promoters was similar to that observed in steady-state growth conditions, although it took somewhat longer to reach the second steady-state of expression when cells were shifted to malate.

Like many other heterotrophic bacteria, the Gram-positive soil bacterium *Bacillus subtilis* can utilize a wide range of sugars, organic acids, and other organic compounds as sources of carbon and energy. Traditionally, glucose was regarded as the preferred carbon source for *B. subtilis*; this view is supported by the rapid growth and by the strong repression of genes encoding the enzymes for the utilization of alternative carbon sources in the presence of glucose (15, 52). Recently, it was discovered that malate serves as a second preferred carbon source for *B. subtilis*; its utilization occurs in parallel with that of glucose, and as observed for glucose, malate causes a strong catabolite repression of transporters for alternative carbon sources (28).

Catabolite repression is the regulatory mechanism that allows the cells to choose among several available carbon sources. Usually, it involves regulation at the level of gene expression to prevent transcription of catabolic genes and operons (catabolite repression in a strict sense) as well as regulation at the level of protein activity to prevent uptake or

formation of the specific inducers of catabolic operons (inducer exclusion) (8, 20). Repression of the *Escherichia coli* lactose operon by glucose was the first example of gene regulation understood at the molecular level, and it is still the paradigm for carbon catabolite repression. However, the molecular mechanisms by which catabolite repression is achieved differ strongly between the different bacteria. In *E. coli*, inducer exclusion and transcription activation of catabolic genes by the complex of the Crp protein with cyclic AMP are crucial (6, 46). In contrast, in *B. subtilis* and most other Gram-positive bacteria, catabolite repression is exerted by the CcpA repressor protein (15, 55).

In many bacteria, glucose and other sugars are transported and concomitantly phosphorylated by the phosphotransferase system (PTS). This system is composed of two soluble general phosphotransferases, enzyme I and HPr, and of a set of sugar-specific multidomain permeases. The domains of the permeases may be present in one protein, or they may exist as individual polypeptides (9). In *B. subtilis* and most other Gram-positive bacteria, HPr is a key player in the regulation of carbon metabolism: this protein can become phosphorylated by enzyme I on His-15 to be active in sugar transfer in the PTS, and in addition it is subject to a regulatory phosphorylation on Ser-46 (9). The phosphorylation of HPr on Ser-46 is catalyzed by the metabolite-sensitive HPr kinase/phosphorylase HPrK (41). The latter phosphorylation has several consequences: (i) the protein is not longer active in glucose transport, since it is a very poor substrate for enzyme I (12); (ii) HPr loses the ability to phosphorylate and thus to activate the PRD-type transcription regulators that are required for the expression of

* Corresponding author. Mailing address for J. Stülke: Department of General Microbiology, Institute of Microbiology and Genetics, Georg-August University Göttingen, Grisebachstr. 8, D-37077 Göttingen, Germany. Phone: 49-551-393781. Fax: 49-551-393808. E-mail: jstuelk@gwdg.de. Mailing address for S. Aymerich: INRA, UMRI1319 Micalis, F-78350 Jouy-en-Josas, France. Phone: 33-1-30815449. Fax: 33-1-30815457. E-mail: stephane.aymerich@grignon.inra.fr.

These authors contributed equally to this work.

§ Supplemental material for this article may be found at <http://jb.asm.org/>.

[∇] Published ahead of print on 14 October 2011.

specific catabolic operons (34); and (iii) HPr(Ser-P) serves as a cofactor for the transcription factor CcpA (10). In the complex with HPr(Ser-P), CcpA is able to bind specific target sites (catabolite-responsive element, or *cre*, sites) in the control region of its target genes (27). This binding results in the repression of a large set of about 300 genes (15). Moreover, transcription of some genes involved in overflow metabolism and amino acid biosynthesis is activated by the CcpA-HPr(Ser-P) complex (22, 35, 42). Thus, the activity of the HPr kinase/phosphorylase is crucial not only for the phosphorylation state of HPr but also for the regulatory output of CcpA. The HPrK is a bifunctional enzyme; its kinase activity is allosterically activated by ATP and fructose 1,6-bisphosphate (FBP), i.e., under conditions of good nutrient supply (26). Under starvation conditions, or when less favorable carbon sources are present, the HPrK has no kinase activity, nor does it even dephosphorylate HPr(Ser-P) if the pool of inorganic phosphate is high (39, 40). Thus, the HPr kinase couples the nutrient state of the cell to the regulatory outcome that is directly governed by CcpA.

B. subtilis is able to use many intermediates of the citric acid cycle as single carbon sources. However, malate seems to have a special position, since it is the only known substrate that can be cometabolized with glucose (28). In order to utilize malate, the bacteria need a functional citric acid cycle and a link to gluconeogenesis. The citric acid cycle and gluconeogenesis are sufficient to provide the cell with all precursors for anabolic reactions. In *B. subtilis*, malate is linked to gluconeogenesis by the activities of malate dehydrogenase and phosphoenolpyruvate carboxykinase. Moreover, four malic enzymes that catalyze the oxidative decarboxylation of malate with the formation of pyruvate are present in *B. subtilis* (13). The mechanisms of malate utilization have not yet been elucidated in detail, but the malate dehydrogenase Mdh and the NADP-dependent malic enzyme YtsJ seem to be very important, since corresponding mutant strains exhibit impaired growth with malate as the only carbon source (32). While the expression of Mdh and YtsJ is constitutive, the phosphoenolpyruvate carboxykinase (PckA) is expressed only when the bacteria grow in the absence of glucose, since the corresponding *pckA* gene is subject to repression by the transcription factor CcpN (50). Once phosphoenolpyruvate is formed, the glycolytic enzymes that catalyze the reversible reactions, the anabolic glyceraldehyde 3-phosphate dehydrogenase GapB (14) and the fructose 1,6-bisphosphatase, lead to the synthesis of glucose 6-phosphate.

While malate was shown to exert efficient catabolite repression of several nutrient transporters, the mechanism(s) involved in this repression has so far remained unknown. It was proposed that the mechanism may differ from the canonical CcpA-HPr(Ser-P) repression pathway, since the concentration of the metabolite that triggers the activity of the HPrK might be too low in the presence of malate as the only carbon source (28). In this work, we demonstrate that the repression by malate is also exerted by the CcpA-HPr(Ser-P) complex. We demonstrate that the complex malate metabolism allows the accumulation of the regulatory metabolites ATP and FBP that are sufficient to trigger the HPrK kinase activity and thus carbon catabolite repression.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *B. subtilis* strains used in this study are listed in Table 1. The presence of the *ptsHI* mutation was verified by sequencing of chromosomal DNA of the relevant strains. *E. coli* DH5 α and *E. coli* TG1 (47) were used for plasmid constructions and transformation using standard techniques (47).

Luria-Bertani (LB) broth was used to grow *E. coli* and *B. subtilis*. When required, media were supplemented with antibiotics at the following concentrations: ampicillin, 100 $\mu\text{g ml}^{-1}$; spectinomycin, 150 $\mu\text{g ml}^{-1}$ (for *E. coli*); spectinomycin, 100 $\mu\text{g ml}^{-1}$; kanamycin, 7.5 $\mu\text{g ml}^{-1}$; chloramphenicol, 5 $\mu\text{g ml}^{-1}$ (for *B. subtilis*).

B. subtilis was grown in C minimal medium supplemented with carbon sources and auxotrophic requirements (at 50 mg liter $^{-1}$) as indicated (54). Potentially repressing carbon sources were used at a concentration of 0.5% (wt/vol) unless stated otherwise. SP plates were prepared by the addition of 17 g liter $^{-1}$ Bacto agar (Difco) to sporulation medium.

Transformation and enzyme assays. Chromosomal DNA of *B. subtilis* was isolated using the DNeasy tissue kit (Qiagen) according to the supplier's protocol. *B. subtilis* was transformed with plasmids and chromosomal DNA according to the two-step protocol (1, 30). Transformants were selected on SP plates containing antibiotics as indicated above or erythromycin plus lincomycin (2 and 25 $\mu\text{g ml}^{-1}$, respectively). For enzyme assays, cells were harvested in exponential growth phase at an optical density at 600 nm (OD $_{600}$) of 0.6 to 0.8. β -Xylosidase activities were determined in cell extracts using *p*-nitrophenyl xyloside as the substrate (33).

In vivo detection of protein-protein interactions. The isolation of protein complexes from *B. subtilis* cells was performed by the SPINE technology (25) using a chromosomally encoded CcpA protein carrying a C-terminal Strep tag. For the construction of this strain, GP1303, we made use of the cloning vector pGP1389, which allows easy integration of the constructs into the chromosome (31). The *ccpA* gene was amplified (for primers, see Table S1 in the supplemental material), and the PCR product was cloned between the BamHI and SalI sites of pGP1389, giving pGP1952. This plasmid was used to construct *B. subtilis* GP1303 expressing the Strep-tagged CcpA. For the purification of CcpA with its interaction partners, growing cultures of *B. subtilis* were treated with formaldehyde (0.6% [wt/vol], 20 min) to facilitate cross-linking of interacting proteins (25). The Strep-tagged CcpA and its potential interaction partners were then purified from crude extracts using a Strep-Tactin column (IBA, Göttingen, Germany) and desthiobiotin as the eluent. HPr was identified by Western blot analysis.

Analysis of the phosphorylation state of HPr in vivo. HPr phosphorylation was assayed *in vivo* by Western blot analysis as follows. Bacteria were grown in C minimal medium with succinate and glutamate (CSE) in the presence of the indicated carbon sources to an OD $_{600}$ of 0.6. Cells were disrupted using a French press, and crude extracts were prepared as described previously (37). Proteins (3.5 μg of each sample) were separated on nondenaturing 12% polyacrylamide gels. On these gels, phosphorylated HPr migrates faster than the nonphosphorylated protein. HPr(His-P) was dephosphorylated by incubation of the crude extract for 10 min at 70°C. After electrophoresis, the proteins were blotted to a polyvinylidene difluoride (PVDF) membrane. The different forms of HPr were detected using antibodies directed against *B. subtilis* HPr (40). The antibodies were visualized by using anti-rabbit IgG-AP secondary antibodies (Chemikon International, Temecula, CA) and the CDP* detection system (Roche Diagnostics).

Determination of FBP concentrations. Protein-free cell extracts for the determination of fructose 1,6-bisphosphate (FBP) concentrations in *B. subtilis* were prepared as described previously (39, 51). Briefly, cells of the *B. subtilis* wild-type strain 168 were grown in 50 ml CSE medium in the presence of the indicated carbon sources. For each growth condition, at least three independent experiments were carried out. Cultures were harvested by centrifugation, and pellets were frozen in liquid nitrogen. The pellets were resuspended in 0.6 M cold perchloric acid and subsequently incubated on ice for 20 min. The precipitated proteins and cell debris were removed by centrifugation. The pH in the supernatant was adjusted to 7.4 with a solution of cold 0.6 M KOH in 100 mM Tris-HCl (pH 7.4). The precipitated KClO $_4$ was removed by centrifugation. The FBP concentrations were determined in the supernatants as described previously (39).

Determination of ATP concentrations. Intracellular ATP levels were determined as described previously (37). Exponentially growing cultures were quenched at an OD $_{600}$ of 0.5 by mixing with dimethyl sulfoxide (DMSO), which released the adenine nucleotides from the cells. ATP levels were measured using an ATP bioluminescence assay kit (CLSII; Roche Diagnostics) and a microplate fluorescence reader (FLUOstar Omega; BMG Labtech). For calculating the

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

Strain	Relevant genotype ^a	Source, construction, or reference ^b
168	<i>trpC2</i>	Laboratory collection
BBA0028	<i>P_{gntR}-gfpmut3/spec</i>	pBSBgntR → BSB168
BBA0118	<i>P_{fruR}-gfpmut3/spec</i>	pBSBfruR → BSB168
BBA0121	<i>P_{glpF}-gfpmut3/spec</i>	pBSBglpF → BSB168
BBA0223	<i>P_{bglP}-gfpmut3/spec</i>	pBSBbglP → BSB168
BBA0359	<i>P_{sacP}-gfpmut3/spec</i>	pBSBsacP → BSB168
BSB168	Wild type	5
GM1038	<i>sacA321 Δ(sacR-B)₂₃ ccpA::Tn917Δ(erm lacZ)::phleo</i>	Laboratory collection ^c
GM1222	<i>trpC2 pheA1 Δ(bgaX) amyE::(gntRK'-lacZ) ptsH1~cat</i>	11
GM2907	<i>ccpA::Tn917Δ(erm lacZ)::phleo</i>	GM1038 → BSB168
GM2924	<i>crh::aphA3</i>	QB7098 → BSB168
GM2933	<i>ptsH1~cat</i>	GM1222 → BSB168
GM3001	<i>P_{araA}-gfpmut3/spec</i>	pBSBaraA → BSB168
GM3002	<i>P_{gntRcre2}-gfpmut3/spec</i>	pBSBgntR _{cre2} → BSB168
GM3003	<i>P_{sacPΔRAT-term}-gfpmut3/spec</i>	pBSBsacP _{ΔRAT-term} → BSB168
GM3004	<i>P_{glpFΔterm}-gfpmut3/spec</i>	pBSBglpF _{Δterm} → BSB168
GM3005	<i>fruA-gfpmut3/spec</i>	pBSBfruA → BSB168
GM3007	<i>P_{bglPΔRAT-term}-gfpmut3/spec</i>	pBSBbglP _{ΔRAT-term} → BSB168
GM3008	<i>P_{araE}-gfpmut3/spec</i>	pBSBaraE → BSB168
GM3013	<i>P_{araA}-gfpmut3/spec ccpA::Tn917Δ(erm lacZ)::phleo</i>	GM3001 → GM2907
GM3014	<i>P_{gntRcre2}-gfpmut3/spec ccpA::Tn917Δ(erm lacZ)::phleo</i>	GM3002 → GM2907
GM3015	<i>P_{sacPΔRAT-term}-gfpmut3/spec ccpA::Tn917Δ(erm lacZ)::phleo</i>	GM3003 → GM2907
GM3016	<i>P_{glpFΔterm}-gfpmut3/spec ccpA::Tn917Δ(erm lacZ)::phleo</i>	GM3004 → GM2907
GM3017	<i>fruA-gfpmut3/spec ccpA::Tn917Δ(erm lacZ)::phleo</i>	GM3005 → GM2907
GM3019	<i>P_{bglPΔRAT-term}-gfpmut3/spec ccpA::Tn917Δ(erm lacZ)::phleo</i>	GM3007 → GM2907
GM3020	<i>P_{araE}-gfpmut3/spec ccpA::Tn917Δ(erm lacZ)::phleo</i>	GM3008 → GM2907
GM3022	<i>P_{glpF}-gfpmut3/spec ccpA::Tn917Δ(erm lacZ)::phleo</i>	BBA0121 → GM2907
GM3023	<i>P_{bglP}-gfpmut3/spec ccpA::Tn917Δ(erm lacZ)::phleo</i>	BBA0223 → GM2907
GM3024	<i>P_{sacP}-gfpmut3/spec ccpA::Tn917Δ(erm lacZ)::phleo</i>	BBA0359 → GM2907
GM3027	<i>P_{fruR}-gfpmut3/spec ccpA::Tn917Δ(erm lacZ)::phleo</i>	BBA0118 → GM2907
GM3029	<i>P_{gntRcre2}-gfpmut3/spec crh::aphA3</i>	GM3002 → GM2924
GM3031	<i>P_{bglP}-gfpmut3/spec crh::aphA3</i>	BBA0223 → GM2924
GM3033	<i>P_{bglPΔRAT-term}-gfpmut3/spec crh::aphA3</i>	GM3007 → GM2924
GM3037	<i>P_{gntRcre2}-gfpmut3/spec ptsH1~cat</i>	GM3002 → GM2933
GM3039	<i>P_{bglP}-gfpmut3/spec ptsH1~cat</i>	BBA0223 → GM2933
GM3043	<i>P_{bglPΔRAT-term}-gfpmut3/spec ptsH1~cat</i>	GM3007 → GM2933
GM3059	<i>fruA-gfpmut3/spec crh::aphA3</i>	GM3005 → GM2924
GM3057	<i>P_{glpFΔterm}-gfpmut3/spec crh::aphA3</i>	GM3004 → GM2924
GM3061	<i>P_{fruR}-gfpmut3/spec crh::aphA3</i>	BBA0118 → GM2924
GM3063	<i>P_{araE}-gfpmut3/spec crh::aphA3</i>	GM3008 → GM2924
GM3065	<i>P_{araA}-gfpmut3/spec crh::aphA3</i>	GM3001 → GM2924
GM3067	<i>P_{sacPΔRAT-term}-gfpmut3/spec crh::aphA3</i>	GM3003 → GM2924
GM3069	<i>P_{glpFΔterm}-gfpmut3/spec ptsH1~cat</i>	GM3004 → GM2933
GM3071	<i>fruA-gfpmut3/spec ptsH1~cat</i>	GM3005 → GM2933
GM3073	<i>P_{fruR}-gfpmut3/spec ptsH1~cat</i>	BBA0118 → GM2933
GM3075	<i>P_{araE}-gfpmut3/spec ptsH1~cat</i>	GM3008 → GM2933
GM3077	<i>P_{araA}-gfpmut3/spec ptsH1~cat</i>	GM3001 → GM2933
GM3079	<i>P_{sacPΔRAT-term}-gfpmut3/spec ptsH1~cat</i>	GM3003 → GM2933
GP270	<i>trpC2 xylR::ermC</i>	51
GP284	<i>trpC2 xylR::ermC ptsH1</i>	51
GP287	<i>trpC2 xylR::ermC ptsH1 crh::spc</i>	51
GP289	<i>trpC2 xylR::ermC hprK::aphA3</i>	51
GP297	<i>trpC2 xylR::ermC crh::spc</i>	51
GP853	<i>trpC2 xylR::ermC ccpA::spc</i>	51
GP1303	<i>trpC2 ccpA-Strep spc</i>	pGP1952 → 168
QB7098	<i>trpC2 crh::aphA3 amyE::(PΔB levD'-lacZ cat)</i>	17

^a *P_{gntRcre2}-gfpmut3* corresponds to a fusion with both *cre* sites, including *cre2* internal to the *gntR* ORF; *P_{sacPΔRAT-term}-gfpmut3*, *P_{bglPΔRAT-term}-gfpmut3*, and *P_{glpFΔterm}-gfpmut3* correspond to fusions without the regulatory region (terminator and RAT sequence, when relevant) and are therefore expressed constitutively.

^b Arrows indicate construction by transformation.

^c GM1038 was derived in several steps from strain TH256, a *ccpA* mutant by Tn917lac transposition obtained by Tina Henkin (57).

concentrations of intracellular ATP, we used the previously reported aqueous cell volume of 0.85 μl of a culture of 1 ml at an OD₆₀₀ of 1 (16).

Plasmid constructions for transcriptional fusions. Fragments corresponding to the promoter regions of *araA*, *araE*, *bglP*, *fruR*, *glpF*, *gntR*, and *sacP*, or to a region in the 3' part of *fruA*, were amplified by PCR from genomic DNA using the appropriate pairs of primers listed in Table S1 in the supplemental material. PCR products were purified after electrophoresis from agarose gel using the

Wizard SV gel and PCR clean-up system (Promega, Madison, WI). For *bglP*, *sacP*, *glpF*, and *gntR*, two types of fragments were synthesized: for the first three, the expression of which is inducible through an antitermination mechanism, the target region of the antiterminator was either included or omitted, and for *gntR*, in the open reading frame (ORF) of which a second *cre* site is present, fragments including or not including this site were obtained. Ligation-independent cloning of each fragment in the pBaSysBioII plasmid was performed as described pre-

viously (5). The resulting plasmids were extracted from *E. coli* and, after verification of the correct sequence of constructions, used to transform *B. subtilis*. Integration of each plasmid through single recombination allowed the generation of a transcriptional *gfp*mut3 fusion with the corresponding promoter, either directly downstream of it (*araA*, *araE*, *bglP*, *fruR*, *glpF*, *gntR*, and *sacP*) or placed downstream from the last gene of the operon (*fruA*).

Analysis of fluorescence in the LCA. In order to optimize monitoring of fluorescence and to avoid as much as possible any interference linked to the culture medium, live cell array (LCA) experiments were performed as previously set up (5). A modified M9 medium was used (24) supplemented with isoleucine (25 mg liter⁻¹), leucine (50 mg liter⁻¹), valine (40 mg liter⁻¹), and methionine (20 mg liter⁻¹) (4). For these experiments, we used strains derived from BSB168, a *trp*⁺ derivative of *B. subtilis* 168, to avoid the intrinsic fluorescence of tryptophan. If not stated otherwise, the primary carbon source was succinate + glutamate (SE) (0.5% each). When necessary for induction, the suitable carbon source (L-arabinose, D-gluconate, D-fructose, glycerol, D-salicin, or D-sucrose) was added at 0.5%. Steady-state and dynamic experiments were performed as previously described (5). Cells were grown in 100 μ l of medium in a 96-well cell culture plate (Cellstar; Greiner Bio-One) and incubated at 37°C under constant shaking in a Synergy II microplate reader (Biotek) for 20 h. The OD₆₀₀ and fluorescence (excitation, 485/20 nm; emission, 528/20 nm) were measured every 10 min (5). Absorbencies at 900 nm and 977 nm (*A*₉₀₀ and *A*₉₇₇, respectively) were read once at the beginning of each experiment in order to correct the OD₆₀₀ to an optical path length of 1 cm. The carbon source shift was carried out by injection of either glucose to a final concentration of 0.3% or malate to a final concentration of 0.5%, when cells reached an OD₆₀₀ of about 0.3. Fluorescein was present on the microtiter plate at two different concentrations (1 and 10 nM) in duplicate. Each culture was performed in triplicate.

Promoter activity and GFP concentration determination. Green fluorescent protein (GFP) accumulation of the GFPmut3 stable variant was obtained as previously described for *B. subtilis* (5). Polynomial and exponential functions were used to fit the experimental data sets of GFP and biomass, respectively, and to deduce the rates of biomass and GFP production along the growth. GFP concentration was estimated as GFP per OD₆₀₀ (GFP/OD) at each time point. Promoter activities were calculated taking the derivative of the fluorescence divided by the OD₆₀₀ [(dGFP/dt)/OD] at each time point (44, 58). Under steady-state growth conditions (μ = constant), both GFP/OD and (dGFP/dt)/OD are constant. GFP concentration was expressed as units per OD₆₀₀ (1 unit being equivalent to 1 pM fluorescein) and promoter activity as units per hour per OD₆₀₀ unit.

RESULTS

Malate represses transcription of several uptake systems of alternative carbon sources. It was recently shown that malate represses the cointegration of alternative substrates in *B. subtilis* (28). In order to further detail the underlying mechanism, we investigated transcription of the genes coding for uptake systems of several different carbon sources (from C₃ to C₁₃) in the presence/absence of malate. We constructed *gfp* transcriptional fusions reporting promoter activities of transporters, either PTS or not, for fructose (FruA), sucrose (SacP), β -glucosides (BglP), arabinose (AraE), gluconate (GntP), and glycerol (GlpF). Strains were first cultivated in batch culture on M9 minimal medium with either succinate/glutamate (M9SE) as the sole carbon source or on M9SE plus the suitable inducer. For all the fusions, *gfp* expression was barely detectable without the suitable inducer and at least >10 times higher in its presence (Table 2). Gfp expression in all reporter strains was then measured in the presence of the suitable inducer and either glucose or malate. Glucose elicited a repression over 70% (Table 2), except for gluconate and fructose uptake genes, for which the repression was moderate (about 45%). In agreement with the hierarchy in carbon catabolite repression (CCR) of carbon sources (51), expression of the transporter of fructose, which is high in the hierarchy, is the least repressed by glucose. Conversely, expression of the transporter of arabi-

TABLE 2. Expression levels of genes coding for transporters and/or assimilation pathways of several alternative substrates in the absence and presence of malate and glucose

Strain, fusion ^b	Expression level ^a		
	M9SE ^c	+ Glucose	+ Malate
BBA0223, P _{bglP} - <i>gfp</i>	<5 ^d	16	31
BBA0359, P _{sacP} - <i>gfp</i>	<5 ^d	31	>90 ^e
BBA0121, P _{glpF} - <i>gfp</i>	ND	17	59
BBA0028, P _{gntR} - <i>gfp</i>	5	55	60
GM3005, P _{fruA} - <i>gfp</i>	<5 ^d	60	86
BBA0118, P _{fruR} - <i>gfp</i>	10	>90 ^e	90
GM3008, P _{araE} - <i>gfp</i>	9	<5 ^d	16
GM3001, P _{araA} - <i>gfp</i>	<5 ^d	8	20

^a Expression levels are given as a percentage of the expression in the presence of the suitable inducer. Mean values of results from three independent experiments with standard deviations of $\leq \pm 10\%$ are presented. ND, not determined.

^b The inducer was D-gluconate for the P_{gntR}-*gfp* strain, D-fructose for the *fruA*-*gfp* and P_{fruR}-*gfp* strains, glycerol for the P_{glpF}-*gfp* strain, salicin for the P_{bglP}-*gfp* strains, sucrose for the P_{sacP}-*gfp* strain, and L-arabinose for the P_{araE}-*gfp* and P_{araA}-*gfp* derivative strains.

^c M9 minimal medium with succinate and glutamate (see Materials and Methods).

^d Expression level was below 5% or negligible.

^e Expression level was between 90 and 100% and was not distinguishable from the induced condition with this assay.

nose, which is low in the hierarchy, is the most repressed (Table 2). Similarly, in all but one case (P_{sacP}-*gfp*), malate triggered repression of the reporter fusions, although at a lower level than that exerted by glucose (Table 2). The weaker repression level together with the absence of measurable repression of the *sacP* promoter may result either from a second, distinct repression mechanism based on the mode of action of the inducer or from an intermediate activation of the HPrK/CcpA CCR pathway.

Malate repression is independent from the induction mechanism and dependent upon CcpA. To know whether the catabolite repression by malate depends on the induction mechanism of the different uptake systems, we constructed another set of reporter fusions in which the target sequences of the induction mechanism have been precisely deleted (see Materials and Methods). The resulting constitutive expression of the P_{bglP} Δ RAT-term-*gfp*, P_{sacP} Δ RAT-term-*gfp*, and P_{glpF} Δ term-*gfp* transcriptional fusions was even higher than the induced expression of the corresponding inducible fusion (data not shown). The addition of glucose, malate, or the cognate inducer to the three modified reporter systems triggered a strong repression of *gfp* expression (>80%, >60%, and >70%, respectively) (Fig. 1A). These results indicate that malate catabolite repression is independent from the induction mechanisms of these uptake systems. They also confirm that glycolytic carbon sources other than glucose can trigger catabolite repression, most probably through the HPrK/CcpA pathway (51).

We then addressed the question of whether malate hijacks the HPrK/CcpA CCR regulatory pathway by determining *gfp* expression of the previous reporter strains in a *ccpA* mutant genetic context. Figure 1A shows that the *ccpA* knockout fully relieved the repression of the P_{bglP} Δ RAT-term-*gfp*, P_{sacP} Δ RAT-term-*gfp*, and P_{glpF} Δ term-*gfp* reporter fusions. This demonstrated that malate-mediated repression is exerted via the CcpA repressor on these target genes. We also investigated the effect of *ccpA* knockout on the repression of the P_{gntR}Cre2-*gfp*, *fruA*-*gfp*, P_{araE}-

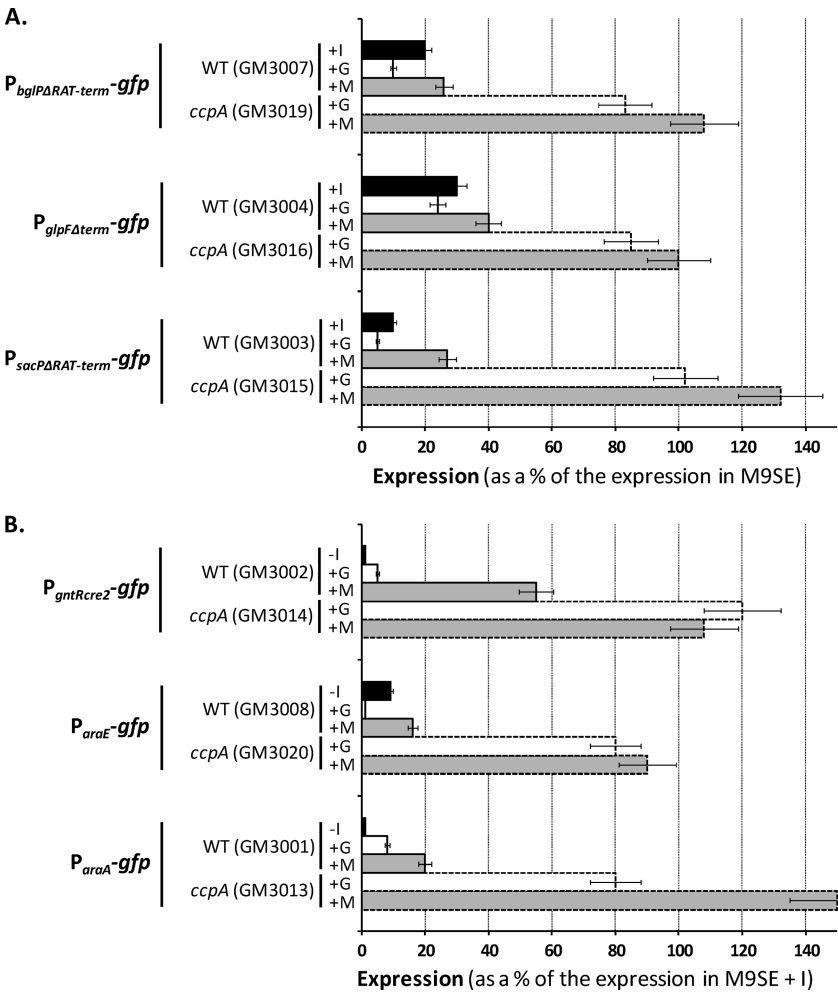


FIG. 1. Carbon catabolite repression in the presence of glucose (+G) or malate (+M). (A) Effect of the *ccpA* inactivation on constitutive fusions. (B) Effect of the *ccpA* inactivation on inducible fusions. Expression levels are given as a percentage of the expression in the presence of the suitable inducer (+I) for fusions requiring induction and of the expression in M9SE for constitutive fusions (–I). Mean values of three independent experiments with standard deviations are presented.

gfp, and $P_{araA}-gfp$ reporter fusions, inducible by their cognate inducer. In the absence of inducer, levels of expression were barely detectable (Fig. 1B). In the presence of the cognate inducer, the addition of glucose or malate did not trigger repression anymore, except for the *fruA-gfp* fusion (data not shown), thus confirming the essential role of CcpA for malate catabolite repression. It is worth noting that the repression exerted by glucose on the $P_{gntRcre2}-gfp$ fusion was stronger than that exerted on the $P_{gntR}-gfp$ fusion, in which the second *cre* site located within the *gntR* coding sequence is absent (15). In order to further investigate the different factors possibly involved in the malate repression, we then focused on the expression of *xynB*, a well-established model system to study the CCR (51).

Roles of factors involved in glucose repression in the repression by malate. To gain further insights into catabolite repression by malate, we analyzed the effect of malate on the synthesis of β -xylosidase, encoded by the *xynB* gene. This gene is subject to a very strong repression by glucose, and in a strain devoid of the xylose repressor XylR, this repression depends exclusively on a functional CcpA regulatory pathway (51).

Therefore, we studied the synthesis of β -xylosidase in the *xylR* mutant strain GP270 after growth in minimal medium without any added carbon source and in the presence of glucose or malate. As shown in Table 3, glucose caused an approximate 25-fold repression of β -xylosidase synthesis. This is in excellent agreement with previous observations (33, 51). In the presence of malate, a 9-fold repression was observed, which led us to address the role of CcpA in malate-mediated repression. As reported previously (51), repression by glucose was nearly completely lost upon inactivation of CcpA. Similarly, repression by malate was strongly reduced in the *ccpA* mutant; however, the β -xylosidase activity in the presence of malate reached only 55% of that observed in CSE medium. This result indicates that CcpA is the major factor causing catabolite repression of β -xylosidase synthesis by malate.

It is well established that CcpA efficiently binds its target sites *in vivo* only if it forms a complex with either of its cofactors, HPr(Ser-P) or Crh(Ser-P). For glucose-mediated catabolite repression of β -xylosidase, the two cofactors can replace each other (17, 19). The formation of these cofactors requires

TABLE 3. Catabolite repression of β -xylosidase by different carbon sources in various mutants

Carbon source ^a	Enzyme activity in units/mg of protein ^b					
	GP270 $\Delta xyIR$	GP853 $\Delta xyIR \Delta ccpA$	GP284 $\Delta xyIR ptsHI$	GP297 $\Delta xyIR \Delta crh$	GP289 $\Delta xyIR \Delta hprK$	GP287 $\Delta xyIR \Delta crh ptsHI$
None (CSE)	1,535 (204)	2,548 (179)	1,535 (91)	2,609 (413)	2,414 (445)	2,644 (321)
Glucose	59 (2)	2,074 (43)	154 (29)	75 (16)	2,036 (209)	2,385 (48)
Malate	170 (27)	1,388 (49)	176 (28)	370 (35)	1,579 (231)	1,598 (95)

^a Added to CSE medium.^b All measurements were performed at least in triplicate. Values in parentheses indicate the standard deviations.

the metabolite-activated HPr kinase, and it was proposed that the concentration of the HPr kinase effector fructose 1,6-bisphosphate (FBP) in malate-grown cells would be too low to allow HPr phosphorylation (28). To address these obvious contradictions, we determined the repression by malate in mutants affected in the formation of the cofactors for CcpA. As observed previously, individual mutations of either HPr (the *ptsHI* mutation results in a loss of the phosphorylation site Ser-46) or Crh did not result in significant changes in repression of β -xylosidase synthesis compared to the use of an isogenic wild-type strain (compare GP270 to GP284 and GP297 in Table 3). As observed for repression of *xynB* expression by glucose, the combination of the *ptsHI* and *crh* mutations resulted in a substantial loss of malate-mediated repression and had essentially the same effect as the *ccpA* mutation (GP287 and GP853 in Table 3). This finding supports the idea that HPr(Ser-P) and Crh(Ser-P) can functionally replace each other as cofactors of CcpA to cause catabolite repression of *xynB* expression. Finally, we tested the effect of a *hprK* mutation. The corresponding mutant strain, GP289, lacks the HPr kinase, and therefore neither HPr nor Crh can be phosphorylated on Ser-46. As expected, both glucose and malate repression of β -xylosidase synthesis were essentially lost, similar to the observations for the *ccpA* and *ptsHI crh* mutant strains.

May Crh-CcpA and HPr-CcpA complexes have different affinities for the same particular *cre* box and therefore favor the binding of either complex to specific *cre* sequences? In the case of *xynB* repression, HPr and Crh replace each other to exert a similar repression. To test whether this is true for other targets of CcpA, we inserted a *crh* or a *ptsHI* mutation in the *gfp* reporter strains described above. As for the β -xylosidase syn-

thesis, the single *ptsHI* mutation weakly altered the level of repression exerted by either glucose or malate, whereas the *crh* mutation did not show any significant phenotype (Table 4). Taken together, these data unequivocally establish that malate-mediated repression is caused by CcpA in a complex with either of its two cofactors, HPr(Ser-P) or Crh(Ser-P).

In vivo interaction of CcpA with HPr during growth with malate. The results described above provide genetic evidence that the interaction of CcpA with HPr(Ser-P) causes catabolite repression of β -xylosidase synthesis during growth with malate. In order to demonstrate this interaction more directly, we attempted to purify CcpA with its interaction partners from cells grown in minimal medium without any additional carbon source and in the presence of glucose or malate. For this purpose, we constructed *B. subtilis* GP1303, which expresses CcpA carrying a C-terminal Strep tag under the control of its native promoter in the chromosome. CcpA was cross-linked *in vivo* to its interaction partners as described in Materials and Methods and purified by affinity chromatography using a Strep-Tactin matrix. The presence of HPr was assayed by Western blot analysis. As shown in Fig. 2, no HPr was detectable when CcpA was purified from CSE-grown cultures. In contrast, HPr was efficiently cross-linked to CcpA when the bacteria were cultivated in the presence of glucose. Similarly, HPr was copurified with CcpA from malate-grown cells. These data demonstrate that the presence of malate is sufficient to trigger the *in vivo* interaction between CcpA and its cofactor, HPr.

Effect of malate on the ATP-dependent phosphorylation of HPr and on the metabolites that control the HPrK activity. Binding of HPr to CcpA requires its prior phosphorylation by

TABLE 4. Repression by malate or glucose in the *ptsHI* and Δcrh single mutants

Fusion	<i>ptsHI</i> genetic context			Δcrh genetic context		
	Strain	Expression level ^a		Strain	Expression level ^a	
		+ Glucose	+ Malate		+ Glucose	+ Malate
P _{bgIPΔRAT-term} -gfp	GM3043	26	27	GM3033	8	30
P _{sacPΔRAT-term} -gfp	GM3079	20	35	GM3067	4	32
P _{gntRcre2} -gfp	GM3037	27	65	GM3029	9	55
P _{glpFΔterm} -gfp	GM3069	45	50	GM3057	20	40
P _{fruA} -gfp	GM3071	72	82	GM3059	55	84
P _{fruR} -gfp	GM3073	80	90	GM3061	>90 ^b	87
P _{araE} -gfp	GM3075	26	9	GM3063	14	20
P _{araA} -gfp	GM3077	39	43	GM3065	5	19

^a Expression level is given as a percentage of the expression in the presence of the suitable inducer for fusions requiring induction and of the expression in M9SE for constitutive fusions. Mean values of three independent experiments with standard deviations of $\pm 10\%$ are presented.^b Expression level comprised between 90 and 100% and was not distinguishable from the induced condition with this assay.

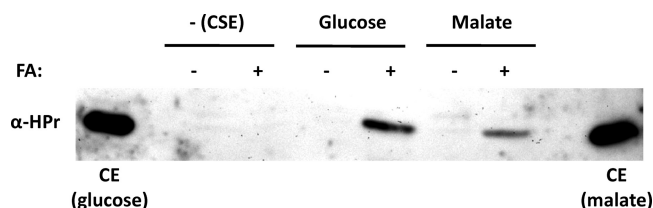


FIG. 2. Detection of *in vivo* interactions between CcpA and HPr by Western blot analysis. The protein complexes were isolated from *B. subtilis* GP1303 with a chromosomally encoded CcpA protein carrying a C-terminal Strep tag. The cells were grown in minimal medium without any repressing carbon source (CSE) or in minimal medium supplemented with glucose or malate. Then, 28 μ l of the elution fractions from each purification without (–FA) or with (+FA) cross-linking by formaldehyde was analyzed by 15% SDS-PAGE. After electrophoresis and blotting onto a PVDF membrane, interaction partners were detected by an antibody raised against HPr. As a control, 3 μ g of the crude extract (CE) from glucose- or malate-grown cells was used.

the HPr kinase at the regulatory site, Ser-46. As shown above, the *hprK* mutant exhibited a strongly reduced repression of β -xylosidase synthesis by malate, suggesting that HPr phosphorylation on Ser-46 is also essential for catabolite repression by malate. To obtain *in vivo* evidence for the hypothesis that HPr is phosphorylated on Ser-46 in malate-grown cells, we determined the phosphorylation pattern of HPr in cells grown in a nonrepressing minimal medium and in the presence of glucose or malate. For this purpose we made use of the different migrations of the HPr forms in native gels. As shown in Fig. 3 (lower panel), the amounts of HPr were similar under all three tested conditions. This is in good agreement with previous observations of the constitutively high expression of HPr (21, 51). In *B. subtilis* grown in CSE medium, HPr was present mainly in the nonphosphorylated form. In addition, a band corresponding to monophosphorylated HPr was visible. This band disappeared after incubation of the cell extract at 70°C. This heat lability is indicative for a phosphorylation on a histidine residue, i.e., His-15 of HPr (40). In the presence of glucose, a large portion of HPr was phosphorylated, and this phosphorylation was heat stable. This indicates that phosphorylation had occurred on Ser-46 and is in perfect agreement with results of previous investigations (37, 40, 51). In the malate-grown cells, we also detected that a major fraction of HPr was phosphorylated on Ser-46. Thus, in the presence of malate, HPr is available as HPr(Ser-P) that is competent for the productive interaction with CcpA that we have observed to take place *in vivo*.

HPr(Ser-P) is synthesized by the metabolite-activated HPr kinase HPrK. The activity of this protein is known to be triggered by FBP and ATP (18, 23, 26, 43). However, it was suggested that the concentrations of these metabolites might not reach the critical concentration when *B. subtilis* grows in the presence of malate (28). To clarify this issue, we determined the intracellular concentrations of FBP and ATP. In cells cultivated in CSE minimal medium, the concentration of FBP was 2.3 mM. In contrast, the FBP concentration was significantly increased in glucose-grown cells (12.7 mM). These results are in good agreement with those of previous studies (39, 51). For malate-grown cells, an intermediate concentration of FBP (5.4 mM) was determined (Table 5). This is in the

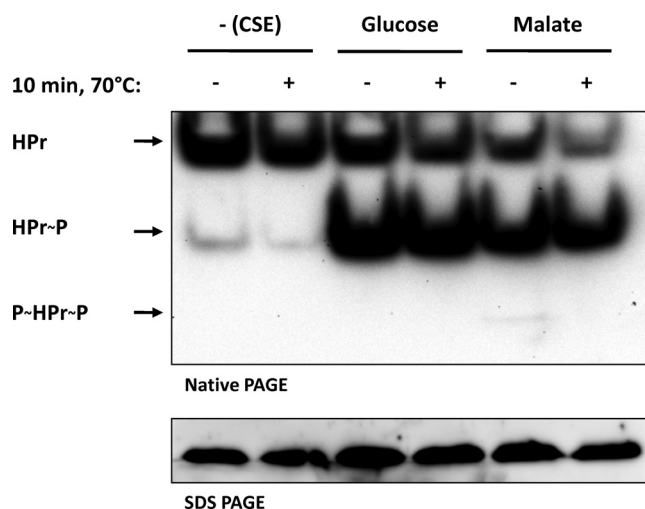


FIG. 3. Determination of the phosphorylation state of HPr in the presence of malate. The wild-type strain 168 was grown in CSE medium or in CSE medium supplemented with glucose or malate. Protein extracts were prepared and separated on native 12% polyacrylamide (PAA) gels (top panel). HPr was subsequently detected by Western blotting. To discriminate between HPr(Ser-P) and HPr(His-P), an aliquot of each cell extract was heated (70°C, 10 min) prior to loading. This causes a loss of the phosphohistidine bonds. The lysis buffer used affected the reliability of the Bradford assay for determination of protein concentrations. To account for the differences in the protein estimation and sample loading, 2 μ g of each protein extract (as determined by the Bradford assay) was separated in parallel by SDS-PAGE, and total HPr was detected by Western blot analysis (bottom panel).

range detected for other repressing carbon sources (51). In contrast to the FBP concentrations, which were quite variable depending on the carbon source, there were only minor differences in the ATP concentrations under the three conditions tested (Table 5). Again, this is in agreement with previous observations for *B. subtilis* (37, 53) and with the idea that the ATP concentrations are generally kept constant in bacterial cells (29).

Dynamic transcriptional response after malate or glucose addition. Although we showed that the level of HPr(Ser-P) was high enough during steady-state growth in the presence of malate to trigger catabolite repression, the FBP level was twofold lower than in the presence of glucose. The lower level of FBP observed in malate-grown cells could explain the weaker repression exerted by malate compared to that by glucose. This weaker FBP concentration may lead to not only lower but also slower dynamics of adaptation to a pulse of malate leading to a slower large-scale reorganization of the regulatory network. We therefore designed shift experiments in which cells were first grown on M9SE before

TABLE 5. Effect of the carbon source on the pools of fructose 1,6-bisphosphate and ATP in *B. subtilis*^a

Carbon source ^a	Fructose 1,6-bisphosphate, mM	ATP, μ M
None (CSE)	2.3 (0.17)	42.6 (4.96)
Glucose	12.7 (0.57)	59.5 (7.52)
Malate	5.4 (0.45)	47.3 (5.14)

^a Added to CSE medium.

^b All measurements were performed at least in triplicate. Values in parentheses indicate the standard deviations.

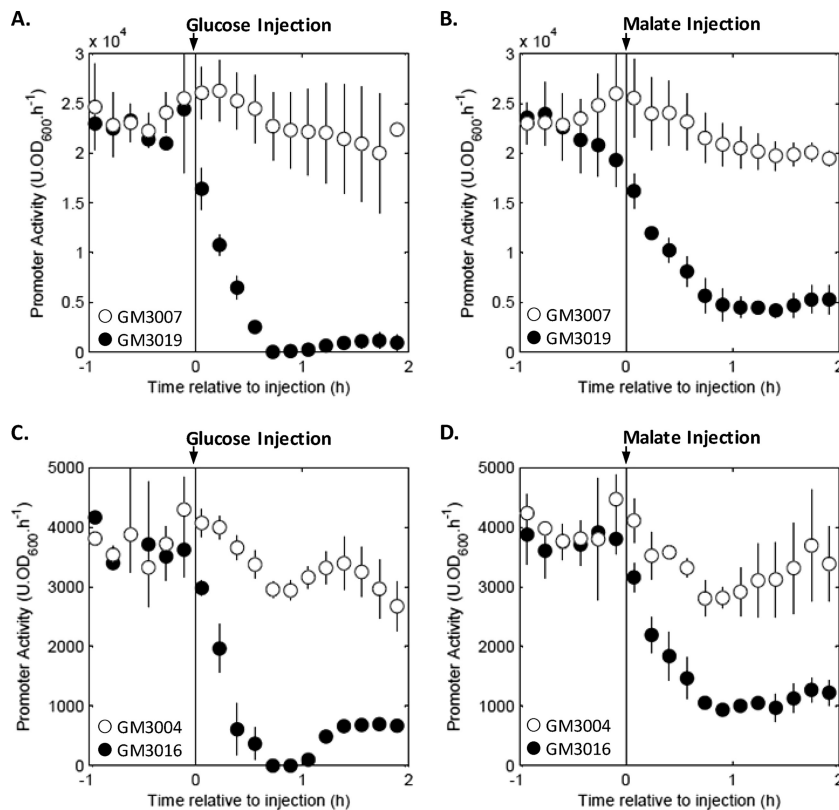


FIG. 4. Carbon catabolite repression upon glucose or malate addition to cells grown under gluconeogenic conditions. Wild-type (black circles) and *ccpA* mutant (white circles) strains were grown on succinate as the sole carbon source until the second substrate addition. The glucose (left panels)- or malate (right panels)-mediated repression was followed using strains bearing the transcriptional fusions $P_{bglP\Delta RAT-term}-gfp$ (strains GM3007 and GM3019; panels A and B) and $P_{glpF\Delta term}-gfp$ (strains GM3004 and GM3016; panels C and D). Repression reached its maximum about 40 min and 50 min after the glucose or malate addition, respectively. Promoter activities are the average results for three replicates, and error bars represent standard deviations.

the addition of glucose or malate. Time course measurement of the expression of the $P_{bglP\Delta RAT-term}-gfp$ reporter fusion during a transition from M9SE to M9SE plus glucose showed that the promoter was fully repressed approximately 40 min after the shift (Fig. 4A). In the following 20 min, the repression was slightly relieved and the promoter activity reached a new plateau corresponding to about 5 to 10% of its level before glucose addition. It is worth noting that the final repression level is similar to that observed under steady-state growth conditions in the presence of glucose (Table 2). As shown in Fig. 4B, the sudden addition of malate elicited a slightly slower repression than that of glucose (maximal repression reached approximately 50 min after the shift). Afterward, the $P_{bglP\Delta RAT-term}-gfp$ promoter activity reached equilibrium at about 25% of the activity prior to malate addition, very similar to that estimated in the malate catabolization steady-state experiments (Table 2). The $P_{sacP\Delta RAT-term}-gfp$ promoter activity behaved dynamically exactly as the $P_{bglP\Delta RAT-term}-gfp$ promoter activity did (data not shown), providing further evidence for the involvement of the identical mechanism in malate catabolite repression of both fusions.

Very similar dynamic expression profiles to those of the previous strains were also observed with the $P_{glpF\Delta term}-gfp$ reporter strain (Fig. 4C and D). Again the stable expression level reached 2 h after the shift was very close to that measured under steady-state growth conditions (Table 2). However, a

transient “overrepression” after glucose addition before reaching a stable level of transcription was even more pronounced with the $P_{glpF\Delta term}-gfp$ reporter strain (Fig. 4C) than with the $P_{bglP\Delta RAT-term}-gfp$ one (Fig. 4A). Transient accumulation of FBP caused by high glucose influx but slower reorganization of the entire central metabolism could provoke this transient overrepression of the reporter fusions.

To determine whether CcpA is also the key element involved in the initial response to glucose or malate addition, we investigated the effect of a *ccpA* knockout on the expression of the reporter fusions during either shifts. As shown in Fig. 4, in such a *ccpA* mutant context, promoter activities of $P_{bglP\Delta RAT-term}-gfp$ and $P_{glpF\Delta term}-gfp$ are no more repressed and remained roughly constant upon glucose or malate addition. The same observation was made with the $P_{sacP\Delta RAT-term}-gfp$ promoter (not shown). Interestingly, both injections onto the *ccpA* mutant-derived strains led to increasing noise and variations in *gfp* expression that may be due to the particular stress caused by a nutritional perturbation in this particular mutant background. Conversely, the *crh* and *ptsHI* single mutants did not show any particular phenotype compared to the wild type (data not shown), suggesting that, as in steady-state growth conditions, Crh and HPr can replace each other. Altogether, these results demonstrated that the HPrK/CcpA pathway transduces the repression signal immediately after malate ad-

dition, as for glucose addition. They also revealed that malate catabolite repression is not only weaker but also slightly slower than glucose catabolite repression, although both malate and glucose maximal repressions were reached in less than a doubling time (see Table S2 in the supplemental material).

DISCUSSION

This work shows that malate causes carbon catabolite repression in *B. subtilis* by employing the general CcpA-dependent mechanism of repression. This underlines the specific role of malate in the physiology of *B. subtilis*. Malate is the primary product of carbon dioxide fixation in a large group of plants, the so-called C_4 plants, as well as in the crassulacean acid metabolism plants. Moreover, malate accumulates in unripe fruits. Thus, malate is commonly available in soil and on plants, the preferred habitats of *B. subtilis*. Indeed, *B. subtilis* has recently been suggested to be an epiphyte (3, 7). Moreover, several carboxylic acids, in particular malate, are substantially secreted by plant roots into the rhizosphere (2), which enables recruiting beneficial bacteria that help to reduce susceptibility to plant pathogen attack (56). The plant pathogen *Pseudomonas syringae* pv. tomato elicits the secretion of malate in *Arabidopsis thaliana*. This malate secretion selectively recruits the beneficial rhizobacterium *B. subtilis* FB17 in a dose-dependent manner (45). With this as the case and with malate as a key nutrient for the bacteria, one would also expect sophisticated mechanisms for malate utilization.

B. subtilis produces five malate dehydrogenases, the canonical Mdh of the citric acid cycle, and four decarboxylating malate dehydrogenases, the so-called malic enzymes. Among the malic enzymes, YtsJ reduces $NADP^+$, whereas the three other enzymes reduce NAD^+ (13, 32). In addition to the Mdh/PckA-dependent conversion of malate to phosphoenolpyruvate, the entry point of gluconeogenesis, the bacteria require a functional *ytsJ* gene for efficient malate utilization (32). Under gluconeogenic conditions (i.e., with malate as the only carbon source), the cells do not have the possibility to produce NADPH by the pentose phosphate pathway. Under glycolytic conditions, about 40 to 50% of the glucose enters this pathway (48, 49), which yields two molecules of NADPH per molecule of glucose. This represents about 50% of the NADPH formation in the cell and is important to provide reducing power for all biosynthetic reactions in the cell. Thus, the conversion of malate to pyruvate using the NADPH-forming malic enzyme YtsJ may be important to balance the redox pool of the cell and to allow efficient anabolic reactions.

The identification of malate as a carbon source that causes catabolite repression immediately raised the question of how this repression might be achieved. In a previous study, a mechanism independent of CcpA/HPr(Ser-P) was postulated based on the presumptive low concentration of fructose 1,6-bisphosphate (28). Here, we provide unequivocal evidence that catabolite repression by malate involves all factors that also play a role in sugar-mediated catabolite transcriptional repression, i.e., CcpA, HPr, Crh, and HPrK. We demonstrate that these factors are required for malate-mediated catabolite repression and that their interactions occur as in the presence of glucose. These interactions include the phosphorylation of HPr by the HPr kinase (as assayed by the *in vivo* HPr phosphorylation

state) and the binding of HPr to CcpA in the presence of glucose or malate. The activity of HPr kinase is the key to carbon catabolite repression in *B. subtilis*, and this activity is mainly controlled by the ATP and the FBP concentrations. At very high ATP concentrations, the enzyme shows kinase activity even in the absence of FBP, but at physiological ATP concentrations FBP acts as an allosteric effector that stimulates kinase activity (26). Both the genetic and the biochemical evidence presented in this work strongly suggested that HPr kinase is active in the presence of malate as the only carbon source. Indeed, the analysis of the ATP and FBP concentrations was in excellent agreement with this idea. It had been shown before that the ATP pool is quite constant in *B. subtilis* even under conditions of different carbon supply (37). In contrast, the FBP concentrations are quite low in media that do not contain repressing carbon sources and are higher in the presence of glucose and other well-metabolizable sugars (39, 51). Here, we demonstrate that an intermediate FBP concentration is present if the bacteria utilize malate. Similar FBP concentrations were observed for sorbitol-, glycerol- or mannitol-grown *B. subtilis* cells. Strikingly, the strength of *xynB* repression exerted by these sugar alcohols is similar to that observed with malate (Table 3) (51). Our results also are in perfect agreement with *in vitro* studies on the activity of HPrK: at an ATP concentration of 25 μ M (i.e., less than detected *in vivo*), 3 mM FBP is sufficient to trigger full HPr kinase activity (26).

In *B. subtilis*, several *cre* boxes were identified either experimentally (about 50) or on the basis of their strong similarity to the consensus sequence (about 100) (15). Repression strength is expected to depend on the affinity of the CcpA-HPr(Ser-P) complex for a particular *cre* box. Repression of *sacP* expression was strong in the presence of either glucose or malate when the sequence involved in the induction mechanism (RAT/terminator) was absent of the reporter fusion (i.e., $P_{sacP\Delta RAT-term}$). This is consistent with the fact that the probable *cre* sequence (TGAAAGCGTATTCT) is highly similar to the consensus (15). However, our results showed no significant repression by malate of *sacP* expression when the reporter fusion contained the full-length wild-type leader region (i.e., P_{sacP}). Besides, repression by glucose of *sacP* expression was also much weaker than that of the constitutive reporter fusion. In addition, the very similar β -glucoside-inducible *bglP* system was more repressed. This suggests that the sucrose-dependent induction mechanism either is strong enough to compensate for the CcpA-mediated catabolite repression or impairs binding of the CcpA-HPr(Ser-P) complex.

Our results indicate that malate is efficiently metabolized and this metabolism generates levels of key intermediates that control catabolite repression similar to those obtained when sugars are utilized. This raises the question how the observed pool of FBP can be reached. The complex pathways of malate metabolism may provide an answer to this question: the Mdh/PckA pathway, which actually forms a physical complex *in vivo* during growth on malate (38), provides the phosphoenolpyruvate that serves as the starting point for gluconeogenesis. In parallel, YtsJ uses malate to generate NADPH. This in turn, is required for the reduction of 1,3-bisphosphoglycerate by the gluconeogenic glyceraldehyde 3-phosphate dehydrogenase GapB. The other gluconeogenic reactions that give rise to FBP

all follow Michaelis-Menten kinetics and the corresponding genes are constitutively expressed (36). In contrast, the *pckA* and *gapB* genes are induced under gluconeogenic conditions (14, 50). In addition, the NAD⁺-dependent malic enzymes might serve for the generation of NADH that can drive oxidative ATP synthesis to provide the cell with energy. Thus, once a sufficient amount of malate becomes available, the concerted activity of the different malate dehydrogenases allows the cell to meet the requirements for precursors, reducing power for anabolism, and energy. Thus, the genetic equipment of *B. subtilis* with enzymes active in malate metabolism reflects the important role of this substrate for the organism and provides a possible explanation of how malate causes carbon catabolite repression.

ACKNOWLEDGMENTS

We thank Bernard Freytag for his contribution to the initial phase of this project and Sabine Lenters for technical assistance. We are grateful to Heinz Neumann for providing access to the FLUOstar Omega fluorescence reader.

This work was supported by grants from the Federal Ministry of Education and Research SYSMO network (PtJ-BIO/0313978D) and the DFG (Hi 291/131) to J.S. as well as by French public funds from the Centre National de la Recherche Scientifique and the Institut National de la Recherche Agronomique and by a grant from the European Community BaSysBio Programme (LSHG-CT-2006-037469) to S.A.

REFERENCES

- Anagnostopoulos, C., and J. Spizizen. 1961. Requirements for transformation in *Bacillus subtilis*. *J. Bacteriol.* **81**:741–746.
- Bais, H. P., T. L. Weir, L. G. Perry, S. Gilroy, and J. M. Vivanco. 2006. The role of root exudates in high-throughput interactions with plants and other organisms. *Annu. Rev. Plant Biol.* **57**:233–266.
- Barbe, V., et al. 2009. From a consortium sequence to a unified sequence: the *Bacillus subtilis* 168 reference genome a decade later. *Microbiology* **155**:1758–1775.
- Bléncke, H.-M., et al. 2003. Transcriptional profiling of gene expression in response to glucose in *Bacillus subtilis*: regulation of the central metabolic pathways. *Metab. Eng.* **5**:133–149.
- Botella, E., et al. 2010. pBaSysBioII: an integrative plasmid generating *gfp* transcriptional fusions for high-throughput analysis of gene expression in *Bacillus subtilis*. *Microbiology* **156**:1600–1608.
- Crasnier, M. 1996. Cyclic AMP and catabolite repression. *Res. Microbiol.* **147**:479–482.
- Deng, Y., et al. 2011. Complete genome sequence of *Bacillus subtilis* BSn5, an endophytic bacterium of *Amorphophallus konjac* with antimicrobial activity for the plant pathogen *Erwinia carotovora* subsp. *carotovora*. *J. Bacteriol.* **193**:2070–2071.
- Deutscher, J. 2008. The mechanisms of carbon catabolite repression in bacteria. *Curr. Opin. Microbiol.* **11**:87–93.
- Deutscher, J., C. Francke, and P. W. Postma. 2006. How phosphotransferase system-related protein phosphorylation regulates carbohydrate metabolism in bacteria. *Microbiol. Mol. Biol. Rev.* **70**:939–1031.
- Deutscher, J., E. Küster, U. Bergstedt, V. Charrier, and W. Hillen. 1995. Protein kinase-dependent HPr/CcpA interaction links glycolytic activity to carbon catabolite repression in Gram-positive bacteria. *Mol. Microbiol.* **15**:1049–1053.
- Deutscher, J., et al. 1994. Loss of protein kinase-catalyzed phosphorylation of HPr, a phosphocarrier protein of the phosphotransferase system, by mutation of the *ptsH* gene confers catabolite repression resistance to several catabolic genes of *Bacillus subtilis*. *J. Bacteriol.* **176**:3336–3344.
- Deutscher, J., U. Kessler, C. A. Alpert, and W. Hengstenberg. 1984. Bacterial phosphoenolpyruvate-dependent phosphotransferase system: P-ser-HPr and its possible regulatory function? *Biochemistry* **23**:4455–4460.
- Doan, T., et al. 2003. The *Bacillus subtilis* *ywkA* gene encodes a malic enzyme and its transcription is activated by the YufL/YufM two-component system in response to malate. *Microbiology* **149**:2331–2343.
- Fillinger, S., et al. 2000. Two glyceraldehyde-3-phosphate dehydrogenases with opposite physiological roles in a non-photosynthetic bacterium. *J. Biol. Chem.* **275**:14031–14037.
- Fujita, Y. 2009. Carbon catabolite control of the metabolic network in *Bacillus subtilis*. *Biosci. Biotechnol. Biochem.* **73**:245–259.
- Fujita, Y., and E. Freese. 1979. Purification and properties of fructose-1,6-bisphosphatase of *Bacillus subtilis*. *J. Biol. Chem.* **254**:5340–5349.
- Galinier, A., et al. 1997. The *Bacillus subtilis* *crh* gene encodes a HPr-like protein involved in carbon catabolite repression. *Proc. Natl. Acad. Sci. U. S. A.* **94**:8439–8444.
- Galinier, A., et al. 1998. New protein kinase and protein phosphatase families mediate signal transduction in bacterial catabolite repression. *Proc. Natl. Acad. Sci. U. S. A.* **95**:1823–1828.
- Galinier, A., J. Deutscher, and I. Martin-Verstraete. 1999. Phosphorylation of either Crh or HPr mediates binding of CcpA to the *Bacillus subtilis* *xyn cre* and catabolite repression of the *xyn* operon. *J. Mol. Biol.* **286**:307–314.
- Görke, B., and J. Stülke. 2008. Carbon catabolite repression in bacteria: many ways to make the most out of nutrients. *Nat. Rev. Microbiol.* **6**:613–624.
- Görke, B., L. Frayse, and A. Galinier. 2004. Drastic differences in Crh and HPr synthesis levels reflect their different impacts on catabolite repression in *Bacillus subtilis*. *J. Bacteriol.* **186**:2992–2995.
- Grundy, F. J., D. A. Waters, S. H. Allen, and T. M. Henkin. 1993. Regulation of the *Bacillus subtilis* acetate kinase gene by CcpA. *J. Bacteriol.* **175**:7348–7355.
- Hanson, K. G., K. Steinhauer, J. Reizer, W. Hillen, and J. Stülke. 2002. HPr kinase/phosphatase of *Bacillus subtilis*: expression of the gene and effects of mutations on enzyme activity, growth and carbon catabolite repression. *Microbiology* **148**:1805–1811.
- Harwood, C. R., and S. M. Cutting. 1990. Chemically defined growth media and supplements, p. 548. In C. R. Harwood and S. M. Cutting (ed.), *Molecular biological methods for Bacillus*. Wiley, Chichester, United Kingdom.
- Herzberg, C., et al. 2007. SPINE: a method for the rapid detection and analysis of protein-protein interactions *in vivo*. *Proteomics* **7**:4032–4035.
- Jault, J. M., et al. 2000. The HPr kinase from *Bacillus subtilis* is a homooligomeric enzyme which exhibits strong positive cooperativity for nucleotide and fructose 1,6-bisphosphate binding. *J. Biol. Chem.* **275**:1773–1780.
- Jones, B. E., et al. 1997. Binding of the catabolite repressor protein CcpA to its DNA target is regulated by phosphorylation of its corepressor HPr. *J. Biol. Chem.* **272**:26530–26535.
- Kleijn, R. J., et al. 2010. Metabolic fluxes during strong carbon catabolite repression by malate in *Bacillus subtilis*. *J. Biol. Chem.* **285**:1587–1596.
- Koebmann, B. J., H. V. Westerhoff, J. L. Snoep, D. D. Nilsson, and P. R. Jensen. 2002. The glycolytic flux in *Escherichia coli* is controlled by the demand for ATP. *J. Bacteriol.* **184**:3909–3916.
- Kunst, F., and G. Rapoport. 1995. Salt stress is an environmental signal affecting degradative enzyme synthesis in *Bacillus subtilis*. *J. Bacteriol.* **177**:2403–2407.
- Lehnik-Habrink, M., et al. 2011. RNase Y in *Bacillus subtilis*: a natively disordered protein that is the functional equivalent of RNase E from *Escherichia coli*. *J. Bacteriol.* **193**:5431–5441.
- Lerondel, G., T. Doan, N. Zamboni, U. Sauer, and S. Aymerich. 2006. YtsJ has the major physiological role of the four paralogous malic enzyme isoforms in *Bacillus subtilis*. *J. Bacteriol.* **188**:4727–4736.
- Lindner, C., J. Stülke, and M. Hecker. 1994. Regulation of xylanolytic enzymes in *Bacillus subtilis*. *Microbiology* **140**:753–757.
- Lindner, C., M. Hecker, D. Le Coq, and J. Deutscher. 2002. *Bacillus subtilis* mutant LicT antiterminators exhibiting enzyme I- and HPr-independent antitermination affect catabolite repression of the *bglPH* operon. *J. Bacteriol.* **184**:4819–4828.
- Ludwig, H., C. Meinken, A. Matin, and J. Stülke. 2002. Insufficient expression of the *ilv-leu* operon encoding enzymes of branched-chain amino acid biosynthesis limits growth of a *Bacillus subtilis* *ccpA* mutant. *J. Bacteriol.* **184**:5174–5178.
- Ludwig, H., et al. 2001. Transcription of glycolytic genes and operons in *Bacillus subtilis*: evidence for the presence of multiple levels of control of the *gapA* operon. *Mol. Microbiol.* **41**:409–422.
- Ludwig, H., N. Rebhan, H. M. Bléncke, M. Merzbacher, and J. Stülke. 2002. Control of the glycolytic *gapA* operon by the catabolite control protein A in *Bacillus subtilis*: a novel mechanism of CcpA-mediated regulation. *Mol. Microbiol.* **45**:543–553.
- Meyer, F. M., et al. 2011. Physical interactions between tricarboxylic acid cycle enzymes in *Bacillus subtilis*: evidence for a metabolon. *Metab. Eng.* **13**:18–27.
- Mijakovic, I., et al. 2002. Pyrophosphate-producing protein dephosphorylation by HPr kinase/phosphorylase: a relic of early life? *Proc. Natl. Acad. Sci. U. S. A.* **99**:13442–13447.
- Monedero, V., et al. 2001. Mutations lowering the phosphatase activity of HPr kinase/phosphatase switch off carbon metabolism. *EMBO J.* **20**:3928–3937.
- Nessler, S., et al. 2003. HPr kinase/phosphorylase, the sensor enzyme of catabolite repression in gram-positive bacteria: structural aspects of the enzyme and the complex with its protein substrate. *J. Bacteriol.* **185**:4003–4010.
- Presecan-Siedel, E., et al. 1999. The catabolite regulation of the *pta* gene as part of carbon flow pathways in *Bacillus subtilis*. *J. Bacteriol.* **181**:6889–6897.
- Reizer, J., et al. 1998. A novel protein kinase that controls carbon catabolite repression in bacteria. *Mol. Microbiol.* **27**:1157–1169.
- Ronen, M., R. Rosenberg, B. I. Shraiman, and U. Alon. 2002. Assigning

- numbers to the arrows: parameterizing a gene regulation network by using accurate expression kinetics. *Proc. Natl. Acad. Sci. U. S. A.* **99**:10555–10560.
45. Rudrappa, T., K. J. Czymmek, P. W. Paré, and H. P. Bais. 2008. Root-secreted malic acid recruits beneficial soil bacteria. *Plant Physiol.* **148**:1547–1556.
 46. Saier, M. H., Jr. 1989. Protein phosphorylation and allosteric control of inducer exclusion and catabolite repression by the bacterial phosphoenolpyruvate: sugar phosphotransferase system. *Microbiol. Rev.* **53**:109–120.
 47. Sambrook, J., and D. Russell. 2001. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
 48. Sauer, U., et al. 1997. Metabolic fluxes in riboflavin-producing *Bacillus subtilis*. *Nat. Biotechnol.* **15**:448–452.
 49. Schilling, O., et al. 2007. Transcriptional and metabolic responses of *Bacillus subtilis* to the availability of organic acids: transcription regulation is important but not sufficient to account for metabolic adaptation. *Appl. Environ. Microbiol.* **73**:499–507.
 50. Servant, P., D. Le Coq, and S. Aymerich. 2005. CcpN (YqzB), a novel regulator for CcpA-independent catabolite repression of *Bacillus subtilis* gluconeogenic genes. *Mol. Microbiol.* **55**:1435–1451.
 51. Singh, K. D., M. H. Schmalisch, J. Stülke, and B. Görke. 2008. Carbon catabolite repression in *Bacillus subtilis*: quantitative analysis of repression exerted by different carbon sources. *J. Bacteriol.* **190**:7275–7284.
 52. Stülke, J., and W. Hillen. 2000. Regulation of carbon catabolism in *Bacillus* species. *Annu. Rev. Microbiol.* **54**:849–880.
 53. Voelker, U., et al. 1995. Separate mechanisms activate sigma B of *Bacillus subtilis* in response to environmental and metabolic stresses. *J. Bacteriol.* **177**:3771–3780.
 54. Wacker, I., et al. 2003. The regulatory link between carbon and nitrogen metabolism in *Bacillus subtilis*: regulation of the *gltAB* operon by the catabolite control protein CcpA. *Microbiology* **149**:3001–3009.
 55. Warner, J. B., and J. S. Lolkema. 2003. CcpA-dependent carbon catabolite repression in bacteria. *Microbiol. Mol. Biol. Rev.* **67**:475–490.
 56. Weisskopf, L., et al. 2008. Spatio-temporal dynamics of bacterial communities associated with two plant species differing in organic acid secretion: a one-year microcosm study on lupin and wheat. *Soil Biol. Biochem.* **40**:1772–1780.
 57. Zalieckas, J. M., L. V. Wray, and S. Fischer. 1999. *trans*-Acting factors affecting carbon catabolite repression of the *hut* operon in *Bacillus subtilis*. *J. Bacteriol.* **181**:2883–2888.
 58. Zaslaver, A., et al. 2006. A comprehensive library of fluorescent transcriptional reporters for *Escherichia coli*. *Nat. Methods* **3**:623–628.