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trans-Acting Factors Affecting Carbon Catabolite Repression of the *hut* Operon in *Bacillus subtilis*

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In *Bacillus subtilis*, CcpA-dependent carbon catabolite repression (CCR) mediated at several *cis*-acting carbon repression elements (*cre*) requires the seryl-phosphorylated form of both the HPr (*ptsH*) and Crh (*crh*) proteins. During growth in minimal medium, the *ptsH1* mutation, which prevents seryl phosphorylation of HPr, partially relieves CCR of several genes regulated by CCR. Examination of the CCR of the histidine utilization (*hut*) enzymes in cells grown in minimal medium showed that neither the *ptsH1* nor the *crh* mutation individually had any affect on *hut* CCR but that *hut* CCR was abolished in a *ptsH1 crh* double mutant. In contrast, the *ptsH1* mutation completely relieved *hut* CCR in cells grown in Luria-Bertani medium. The *ptsH1 crh* double mutant exhibited several growth defects in glucose minimal medium, including reduced rates of growth and growth inhibition by high levels of glycerol or histidine. CCR is partially relieved in *B. subtilis* mutants which synthesize low levels of active glutamine synthetase (*glnA*). In addition, these *glnA* mutants grow more slowly than wild-type cells in glucose minimal medium. The defects in growth and CCR seen in these mutants are suppressed by mutational inactivation of TnrA, a global nitrogen regulatory protein. The inappropriate expression of TnrA-regulated genes in this class of *glnA* mutants may deplete intracellular pools of carbon metabolites and thereby result in the reduction of the growth rate and partial relief of CCR.

During growth in the presence of rapidly metabolized carbon sources, carbon catabolite repression (CCR) inhibits the utilization of carbon compounds which support low rates of growth. Transcription of the *Bacillus subtilis* histidine utilization operon (*hut*) is regulated in response to carbon availability by the global regulatory proteins CodY (33) and CcpA (17). The CodY repressor protein exerts a low level of CCR at the $hutO_A$ site (previously called $hutO_{CR1}$) located immediately downstream of the *hut* promoter (9). Although the metabolic signal regulating CodY activity is not known, CodY regulates *dpp* and *hut* expression in response to growth rate (9). The highest levels of CodY-dependent repression occur in cells growing rapidly in media containing amino acids. In cells growing with preferred carbon and nitrogen sources (but no amino acids), only a low level of CodY-mediated repression occurs. Since limitation of growth by carbon availability almost completely abolishes CodY-dependent regulation, CodY mediates a low level of CCR at the *dpp* and *hut* promoters (9).

CCR of *hut* expression is mediated primarily by CcpA-dependent repression at the *hut* carbon repression element (*cre*) (previously called $hutO_{CR2}$) centered 209.5 nucleotides downstream of the *hut* transcriptional start site (28, 40). The in vitro binding of CcpA to *cre* sites is stimulated by seryl-phosphorylated HPr [HPr(ser-P)] (13, 16, 18, 22), a phosphocarrier component of the phosphoenolpyruvate-carbohydrate phosphotransferase system (PTS) (29). HPr can be phosphorylated at two residues, His-15 by phosphoenolpyruvate-dependent PTS enzyme I and Ser-46 by the ATP-dependent HprK kinase (15, 30, 31). The *ptsH1* mutation replaces the Ser-46 residue of HPr with alanine and results in the production of a mutant form of HPr that cannot be phosphorylated by HprK (6, 15, 30, 31). CCR of *acsA* (43), *bglPH* (19), *gnt* (6, 22), *iol* (15), *lev* (21), and *xyl* (5) expression is partially relieved in *ptsH1* mutants. The Crh protein, a homolog of HPr, can also be phosphorylated by the HprK kinase and is involved in CCR (14). In a *ptsH1 crh* double mutant, carbon regulation of *acsA*, *ackA*, *iol*, *lev*, and β-xylosidase expression is abrogated (14, 36, 43). Although it has not yet been demonstrated in vitro, the seryl-phosphorylated form of Crh, Crh(ser-P), has been proposed to also function as a corepressor for CcpA (14, 15). CcpB, a homolog of CcpA, has also been reported to control CCR of *gnt* expression during growth with low levels of aeration and during growth on solid medium (3).

HPr(ser-P) and Crh(ser-P) may not be the only CcpA corepressors. The observation that glucose-6-phosphate (Glc-6-P) enhances the in vitro binding of the *Bacillus megaterium* CcpA protein to the *B. megaterium xyl cre* site and of the *B. subtilis* CcpA protein to the downstream *cre* site in the *B. subtilis gnt* operon raises the possibility that Glc-6-P serves as a corepressor for CcpA binding to *cre* sequences in gram-positive bacteria (16, 22). This hypothesis is supported by the finding that inactivation of the gene encoding glucose kinase partially relieves CCR in *Staphylococcus xylosus* (37) and *B. megaterium* (34). Since glucose kinase catalyzes the conversion of glucose (transported by PTS-independent systems) to Glc-6-P, smaller intracellular pools of Glc-6-P may be present in glucose kinase mutants than in wild-type cells during growth with glucose. An additional cofactor, NADP, has been shown to stimulate the binding of the CcpA–HPr(ser-P) complex to the *amyE cre* sequence and increase the ability of CcpA to inhibit transcription (18). The mechanism responsible for the NADP-dependent stimulation of CcpA in vitro activity is not known.

CCR of gene expression is also deficient in *B. subtilis glnA* mutants that synthesize no or low levels of active glutamine synthetase (GS) (10). This class of *glnA* mutants has a pleiotropic phenotype that includes altered regulation of nitrogen and carbon metabolism and growth defects in minimal medium (10). While the defect in the regulation of carbon metabolism in these mutants is not understood, several lines of evidence

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

Strain	Genotype[a]	Source, reference, and/or derivation[b]
168	*trpC2*	This laboratory
QB7097	*trpC2 crh*::*spc*	I. Martin-Verstraete
GM1222	*trpC2 pheA1* Δ(*bgaX*) *ptsH1*(*cat*) Δ*amyE*:: (*gntRK′-lacZ*)	6
JZ4	*trpC2 crh*::*spc*	168 × QB7097 DNAs
JZ5	*trpC2 ptsH1*(*cat*)	168 × GM1222 DNAs
JZ6	*trpC2 glcK*::*spc*	43
JZ7	*trpC2 ccpB*::*spc*	43
TH256	*ccpA*::Tn*917lac lys-3 trpC2 metB10*	T. Henkin
SF168CP	*trpC2 ccpA*::Tn*917lac*	168 × TH256 DNAs
SF168CPS	*trpC2 ccpA*::Tn*917lac*Δ(*lacZ erm*)::*spc*	SF168CP × p917::Sp DNAs, 35
SF62	*trpC2 tnrA62*::Tn*917*	38
SF168A	*trpC2* Δ*glnA*::*spc*	168 × pGLN14 DNAs, 38
SF62A	*trpC2 tnrA62*::Tn*917* Δ*glnA*::*spc*	SF62 × pGLN14 DNAs 38
SF10	Wild type	10
SF22	*glnA22*	10

[a] The genotype symbols are those of Biaudet et al. (2) with the addition of *crh* (14), *tnrA* (38), and *glcK* (32).

[b] Strains were derived by transforming the first strain listed with DNA from the second strain or the plasmid listed.

indicate that GS acts as a sensor of nitrogen availability in *B. subtilis*. The TnrA regulatory protein controls gene expression in response to nitrogen availability (38). The TnrA protein is active during nitrogen-limited conditions, where it positively regulates the expression of some genes and negatively regulates the expression of other genes (12, 38). Since TnrA-activated genes are expressed constitutively in this class of *glnA* mutants, it has been proposed that GS produces or transmits an inhibitory regulatory signal to TnrA during growth with excess nitrogen (38).

In this report, examination of *hut* expression in wild-type and mutant strains grown in minimal medium revealed that CCR of *hut* expression requires either Crh or HPr(ser-P) but not glucose kinase or CcpB. In contrast, HPr is required for *hut* CCR in cells grown in Luria-Bertani (LB) medium. Since mutational inactivation of *tnrA* suppresses the defects in *hut* CCR and growth seen in a Δ*glnA* mutant, GS is not directly involved in the mechanism of *hut* CCR in *B. subtilis*.

MATERIALS AND METHODS

Bacterial strains. Table 1 lists *B. subtilis* strains used in this study. All *lacZ* transcriptional fusions were transformed into strain 168 (*trpC2*) with plasmid DNA as previously described (40). The *tnrA62*::Tn*917* insertion (38) was transferred by transformation with selection for transposon-encoded erythromycin resistance. Transformation with selection for spectinomycin (*spc*) resistance was used to transfer the Δ*glnA*::*spc* (38), *glcK*::*spc* (43), *crh*::*spc*, *ccpA*::Tn*spc*, and *ccpB*::*spc* (43) mutations. The *ptsH1* mutation (6) was transferred by transformation with selection for the genetically linked chloramphenicol resistance gene. Transformants containing the *ptsH1* mutation were identified by their lack of growth on mannitol minimal medium plates containing ammonium as the nitrogen source.

Cell growth, media, and enzyme assays. The methods used for bacterial cultivation have been previously described (1). Liquid minimal cultures were grown in the morpholinepropanesulfonic acid (MOPS) minimal medium of Neidhardt et al. (26). Glucose was added at 0.5% to MOPS minimal medium and at 1% to LB medium (27). Lactate and citrate were added to a final concentration of 0.2% to MOPS minimal medium. All nitrogen sources were added at 0.2% to MOPS minimal medium. Histidase was induced by addition of L-histidine to 0.1% in the growth medium.

Extracts for enzyme assays were prepared as previously described (1) from cells harvested during exponential growth (70 to 90 Klett units). Histidase and β-galactosidase were assayed as previously described (1). β-Galactosidase activity was always corrected for endogenous β-galactosidase activity present in *B. subtilis* 168 cells containing the promoterless *lacZ* gene from pSFL6 or pSFL7 integrated at the *amyE* site. The assay for gluconate kinase has been described previously (11).

Uptake of α-methyl glucoside. α-Methyl glucoside uptake was assayed in cells grown to mid-log phase in MOPS minimal medium containing glucose and glutamine as the carbon and nitrogen sources, respectively. For one generation prior to harvesting, the cultures were grown in the presence of 1% glycerol. Cells were harvested by filtration on a 0.45-μm-pore-size filter (Millipore) and washed with buffer A (MOPS minimal salts containing 0.2% glutamine and 1% glycerol). The washed cells were resuspended in buffer A by shaking at 26°C. Uptake was initiated by the addition of ^{14}C-labeled α-methyl glucoside (New England Nuclear) to a final concentration of 100 μM, which yielded a specific activity of 2 Ci of ^{14}C per mol. Samples of 0.5 ml were removed and filtered through 0.45-μm-pore-size filters which had been presoaked in buffer A. The filters were washed with 10 ml of buffer A containing 100 μM α-methyl glucoside and counted in Ecolite(+) (ICN) scintillation fluid. The uptake rate was determined from the initial linear portion of the uptake curve.

***lacZ* fusions.** pSFL6 and pSFL7 are neomycin-resistant *lacZ* transcriptional fusion vectors that integrate into the *amyE* locus and contain promoterless *trpA-lacZ* and *spoVG-lacZ* genes, respectively (42). The TMS922 *lacZ* fusion contains the *tms* promoter (24) cloned into pSFL7 (42). The HUT924 *lacZ* fusion is a derivative of pSFL7 that contains a DNA fragment with *hut cre* located downstream of the *tms* promoter (42). The HUT646 and BGL2 *lacZ* fusions contain the *B. subtilis hut* and *bglPH* promoters, respectively, cloned into pSFL6 (42). The NRG407 fusion contains the *nrgAB* promoter cloned into pSFL7 (41).

RESULTS AND DISCUSSION

Role of HPr and Crh in CCR of *hut* expression in cells grown in minimal medium. To determine whether HPr and Crh are involved in CCR of *hut* expression, we examined the expression of histidase, the first enzyme in the histidine-degradative pathway, in *B. subtilis* strains containing the *ptsH1* and *crh* mutations. Because no alteration in CCR of histidase expression was seen in strains containing either the *ptsH1* or the *crh* mutation during growth in glucose minimal medium (data not shown), either Crh or HPr is sufficient for *hut* CCR under these growth conditions. CCR of histidase expression could not be evaluated in a *ptsH1 crh* double mutant because addition of histidine to 0.1% in glucose minimal medium (which is required for the induction of the *hut* operon) completely inhibited growth of the *ptsH1 crh* double mutant. To circumvent the histidine-sensitive phenotype of the *ptsH1 crh* double mutant, the *crh* and *ptsH1* mutations were transferred into *B. subtilis* strains containing the HUT646 (*hut-lacZ*) fusion (42). The HUT646 *lacZ* fusion contains the *hut* promoter and downstream *cre* but lacks the downstream transcriptional terminator involved in histidine-dependent *hut* induction (39). As a result, high-level β-galactosidase expression occurs from this fusion in cells grown in the absence of histidine.

In cells grown in glucose minimal medium, β-galactosidase expression from the HUT646 *lacZ* fusion is repressed to similar levels in wild-type, *ptsH1*, and *crh* strains (Table 2). In contrast, CCR of the HUT646 *lacZ* fusion is completely relieved when the *ptsH1 crh* double mutant is grown in minimal medium with glucose as the carbon source (Table 2). The latter result agrees with published results showing that both the *ptsH1* and *crh* mutations are required for complete relief of CCR of *acsA*, *iol*, *lev*, and β-xylosidase expression in cells grown in glucose minimal medium (14, 43). However, the observation that wild-type levels of *hut* CCR are seen in the *ptsH1* mutant was surprising because the *ptsH1* mutation was previously shown to partially relieve CCR of *acsA*, *gnt*, and *bglPH* expression in cells grown in minimal medium (19, 22, 43). This difference is not due to our growth medium or conditions because we also found that the *ptsH1* mutation partially relieves CCR of the expression of β-galactosidase from the *bglPH-lacZ* BGL2 fusion (Table 2) and of gluconate kinase, the first enzyme in the gluconate-degradative pathway (data not shown). The observation that during growth in minimal medium the *ptsH1* mutation does not affect *hut* CCR but that it partially relieves *bglPH* and *gnt* CCR indicates that some *cre* sites are more sensitive to CCR mediated by HPr than by Crh.

TABLE 2. β-Galactosidase levels of *lacZ* fusions in wild-type and mutant strains grown in minimal medium

lacZ fusion[a]	Relevant genotype	β-Galactosidase sp act (U/mg of protein) in cells grown on[b]:		Glucose repression ratio[c]
		Glucose	Citrate	
HUT646	Wild type	0.9	11.6	13
	ptsH1	0.7	11.4	16
	crh	1.0	19.4	19
	crh ptsH1	15.4	19.0	1.2
	glcK	1.2	12.0	10
HUT924	Wild type	2.2	28.8	13
	ptsH1	3.1	29.1	9.4
	crh	2.5	39.9	16
	crh ptsH1	27.1	41.9	1.5
BGL2	Wild type	1.3	22.7	17
	ptsH1	4.2	20.7	4.9

[a] All strains were derivatives of strain 168 and had the indicated *lacZ* fusion integrated as a single copy at the *amyE* locus.

[b] Data are the averages of three or more determinations, which did not vary by more than 15%. Cultures were grown in MOPS minimal medium containing glutamine as the nitrogen source and the indicated carbon source.

[c] The glucose repression ratio was calculated by dividing the enzyme activity found in the culture grown with citrate by the enzyme activity found in the culture grown with glucose.

One possible explanation is that complexes of CcpA with HPr (ser-P) and Crh(ser-P) assume different conformations and that the CcpA–HPr(ser-P) complex has a higher affinity for the *bglPH* and *gnt cre* sequences than the CcpA–Crh(ser-P) complex but that both complexes bind with similar affinities to the *hut cre* sequence.

Role of HPr in CCR of *hut* expression in LB medium-grown cells. Although the *ptsH1* mutation only partially relieves CCR of *gnt* expression in cells grown in minimal medium, no CCR of *gnt* expression occurs when the *ptsH1* mutant is grown in LB medium (6). To determine whether the wild-type *ptsH* gene is required for CCR of *hut* expression in cells grown in LB medium, we examined CCR of *hut* expression in LB medium-grown cells. The HUT924 (P_{tms}-*hut cre-lacZ*) fusion (42) was used in these experiments instead of the HUT646 *lacZ* fusion because expression of the *hut* promoter is severely repressed by the CodY regulatory protein during rapid growth in the presence of amino acids (9). In the HUT924 fusion, *hut cre* is cloned between the unregulated *tms* promoter and *lacZ*. CCR of HUT924 expression is dependent upon the *hut cre* site because expression of the TMS922 (P_{tms}-*lacZ*) fusion, which contains the *tms* promoter transcriptionally fused to *lacZ*, is not regulated by CCR in cells grown in either minimal or LB medium (Table 3 and data not shown). β-Galactosidase expression from the HUT646 and HUT924 fusions is subject to similar levels of CCR in wild-type cells and *ptsH1*, *crh*, and *ptsH1 crh* mutant cells grown in minimal medium (Table 2). Thus, the HUT924 fusion can be used to monitor *cre*-dependent *hut* CCR in LB medium-grown cells.

During growth in LB medium, β-galactosidase expression from the HUT924 (P_{tms}-*hut cre-lacZ*) fusion was repressed 4.7-fold by glucose in wild-type cells while no CCR occurred in the *ptsH1* mutant strain (Table 3). The *ptsH1* mutation also completely relieved CCR of BGL2 (*bglPH-lacZ*) (Table 3) and ASC7 (*acsA-lacZ*) (data not shown) during growth of these strains in LB medium. Interestingly, the HUT924, BGL2, and ACS7 fusions exhibited lower levels of CCR in wild-type cells grown in LB medium than in those grown in minimal medium (Tables 2 and 3 and data not shown).

It is difficult to explain why CCR of *hut*, *gnt*, and *bglPH* expression is completely dependent upon HPr in cells grown in LB medium but not in minimal medium. These results suggest that HPr, but not Crh, is phosphorylated in LB medium-grown cells. However, phosphorylation of HPr and Crh both in vivo and in vitro has been shown to be dependent on the same protein kinase, HprK (15, 30). To explain the difference in the HPr and Crh dependence of CCR during growth in LB and minimal media, it is necessary to postulate that factors in addition to HprK are involved in phosphorylation of these two proteins. One possibility is that the relative phosphorylation states of HPr and Crh are controlled by phosphatases specific for either HPr(ser-P) or Crh(ser-P). If the activities of the HPr(ser-P) and Crh(ser-P) phosphatases are regulated by different metabolites, differences in the levels of these metabolites in cells grown in LB and minimal media might result in the presence of only HPr(ser-P) in LB medium-grown cells. Since the doubling time of wild-type cells in LB medium plus glucose (20 to 25 min) is lower than in glucose-glutamine minimal medium (45 to 70 min), different metabolite levels may be present in wild-type cells grown in these two media. Decreased levels of HPr(ser-P) together with the absence of Crh(ser-P) in LB medium-grown cells may also explain the lower levels of *hut* and *bglPH* CCR seen in cells grown in LB medium than in cells grown in glucose minimal medium.

Growth defects of the *ptsH1* and *crh* mutant strains. Compared to the wild-type, *ptsH1* mutant, or *crh* mutant strain, the *ptsH1 crh* double mutant exhibited several growth defects in minimal medium. The *ptsH1 crh* mutant grew more slowly in glucose minimal medium than wild-type cells. For example, when the glucose minimal medium contained glutamine as the sole nitrogen source, the doubling time of *ptsH1 crh* cultures (80 min) was higher than the doubling time of wild-type, *ptsH1*, and *crh* cultures (60 min). In contrast, we observed no significant difference in the growth rates of wild-type and *ptsH1 crh* cells in glutamine minimal medium containing either malate or citrate as the sole carbon source (data not shown). Furthermore, growth of the *ptsH1 crh* double mutant in minimal and LB media was severely inhibited in the presence of 0.4% glycerol.

Since it has been proposed that CCR mediated by CcpA

TABLE 3. β-Galactosidase levels from *lacZ* fusions in wild-type and mutant strains grown in LB medium

lacZ fusion[a]	Relevant genotype	β-Galactosidase sp act (U/mg of protein) in cells grown in LB medium[b]:		Glucose repression ratio[c]
		Without glucose	With glucose	
TMS922	Wild type	44.0	36.6	1.2
	ptsH1	33.6	33.5	1.0
HUT924	Wild type	21.3	4.5	4.7
	ptsH1	20.6	20.0	1.0
BGL2	Wild type	14.2	2.2	6.5
	ptsH1	15.2	10.5	1.4

[a] All strains were derivatives of strain 168 and had the indicated *lacZ* fusion integrated as a single copy at the *amyE* locus.

[b] Data are the averages of three or more determinations, which did not vary by more than 25%. Cultures were grown in LB medium with or without glucose.

[c] The glucose repression ratio was calculated by dividing the enzyme activity found in the culture grown without glucose by the enzyme activity found in the culture grown with glucose.

TABLE 4. Carbon catabolite repression of histidase expression in wild-type, *tnrA*, and Δ*glnA* strains

Relevant genotype[a]	Value for cells grown on[b]:			
	Glucose		Citrate	
	Doubling time (min)	Histidase sp act (U/mg of protein)	Doubling time (min)	Histidase sp act (U/mg of protein)
Wild type	70	3.3	102	180
tnrA	75	3.4	100	256
Δ*glnA*	101	54.5	NG[c]	
tnrA Δ*glnA*	74	3.1	107	184

[a] All strains were derivatives of strain 168.
[b] Histidase levels are the averages of two or more determinations, which did not vary by more than 25%. Cultures were grown in MOPS minimal medium containing glutamine as the nitrogen source and the indicated carbon source. Histidine was added to induce *hut* expression.
[c] NG, no growth. The Δ*glnA* strain grows only for several generations before entering stationary phase in this medium.

requires HPr(ser-P) and Crh(ser-P), any growth defects caused by the relief of CCR in the *ptsH1 crh* mutant should also be observed with a *ccpA* mutant. Although the growth phenotypes of these two strain are similar, there are differences between the *ccpA* and *ptsH1 crh* mutants. High levels of histidine completely inhibit growth of the *ptsH1 crh* mutant but only partially inhibit growth of the *ccpA* mutant (40). Similarly, high levels of glycerol severely inhibit growth of the *ptsH1 crh* mutant but not the *ccpA* mutant. The *ccpA* mutant is unable to grow on glucose minimal medium containing ammonium as the nitrogen source (40), but the *ptsH1 crh* mutant is able to grow on this medium, with a higher doubling time than that seen with wild-type cells. These differences suggest that CcpA and/or the HPr and Crh proteins have additional independent roles in regulating *B. subtilis* cellular metabolism. The growth phenotype of the *ccpA* and *ptsH1 crh* mutants explains why CCR of *hut* expression, which is subject to carbon regulation by both CcpA and CodY, is completely relieved in the *ccpA* mutant and the *ptsH1 crh* double mutant. Since CodY regulation is responsive to growth rate, no CodY-dependent regulation is expected to occur in the *ccpA* and *ptsH1 crh* mutants, which exhibit growth defects.

Role of GlnA and TnrA in CCR of *hut* expression. CCR is partially relieved in Gln$^-$ *B. subtilis* mutants which synthesize no or low levels of active GS (10). Several lines of evidence indicate that the altered CCR seen in these Gln$^-$ mutants may result from the defective utilization of glucose. First, measurement of intracellular metabolite pools in wild-type and *glnA22* mutant cells grown with excess glucose showed that the pool sizes of Glc-6-P, pyruvate, and 2-ketoglutarate are smaller in the *glnA22* mutant than in wild-type cells (10). The pool sizes of these glycolytic intermediates are decreased in wild-type cells grown with limiting amounts of glucose (8). Second, the doubling time of this class of Gln$^-$ mutants in glutamine minimal medium containing excess glucose as the carbon source (101 min) is higher than that of wild-type cells (70 min) (Table 4) (10). The observation that the level of Glc-6-P is reduced in this class of Gln$^-$ mutants suggested that glucose transport might be impaired. This possibility was tested by examining the uptake of the glucose analog α-methyl glucoside in wild-type and Gln$^-$ cells grown in minimal medium containing glucose and glutamine as the carbon and nitrogen sources. Since similar rates of α-methyl glucoside uptake were seen in wild-type SF10 (1.25 nmol per min per mg) and SF22 (*glnA22*) (1.20 nmol per min per mg) cells, no defect in glucose transport appeared to be present in this Gln$^-$ mutant.

The global nitrogen regulatory protein TnrA activates the expression of many genes during nitrogen-limited growth of *B. subtilis* (38). Unexpectedly, mutational inactivation of *tnrA* was found to suppress the growth defect of a Δ*glnA* mutant in glucose minimal medium (Table 4). To determine whether the *tnrA* mutation also restores CCR to wild-type levels in the Δ*glnA* mutant, histidase expression was examined in the wild type and in Δ*glnA* and *tnrA* mutant cells. In glucose-grown cultures, histidase levels were 16-fold higher in extracts of Δ*glnA* cells than in extracts of wild-type or *tnrA* mutant cells (Table 4). In contrast, histidase levels in the Δ*glnA tnrA* double mutant were similar to the histidase levels in wild-type or *tnrA* mutant cells. Although the Δ*glnA* strain grew for only two generations in citrate minimal medium before growth ceased, the Δ*glnA tnrA* strain grew like the wild-type and *tnrA* strains in minimal medium with citrate as the carbon source (Table 4).

Since mutational inactivation of *tnrA* suppresses the CCR and growth phenotype of this class of *glnA* mutants, the defects in growth and CCR seen in the Δ*glnA* mutant appear to result from the inappropriate expression of TnrA-regulated genes. It is possible that constitutive expression of TnrA-regulated genes in the Δ*glnA* mutant results in a low rate of cellular growth and relief of CCR due to the depletion of intracellular pools of carbon metabolites. For example, the expression of xanthine dehydrogenase, a key enzyme in purine catabolism, is nitrogen regulated by the GS-dependent nitrogen signal pathway in *B. subtilis* (4). Since this signal pathway is defective in Δ*glnA* mutants, purine degradation may occur constitutively in these mutants and result in a futile cycle of purine synthesis and degradation that depletes intracellular carbon pools. If TnrA activates the expression of xanthine dehydrogenase, this futile cycle would not occur in the Δ*glnA tnrA* mutant. Thus, because the defects in growth and CCR seen in the Δ*glnA* mutant are the indirect result of the Δ*glnA* mutation, GS does not directly participate in the mechanism of CCR of *hut* expression in *B. subtilis*.

TnrA-dependent regulation of gene expression in a *ccpA* mutant. The growth defects seen in the *ccpA* and *ptsH1 crh* mutants in glucose minimal medium may result from altered expression of CCR-regulated genes. However, because constitutive expression of TnrA-regulated genes causes a growth phenotype in *glnA* mutants, it is possible that inappropriate expression of TnrA-dependent genes impairs the growth of *ccpA* and *ptsH1 crh* mutants. The latter possibility was tested by examining the expression of the (*nrgAB-lacZ*)*407* fusion in wild-type and *ccpA* mutant cells grown in glucose minimal medium with an excess (glutamine) or a limited (glutamate) nitrogen source. Transcription from the *nrgAB* promoter occurs only during nitrogen-limited conditions and is completely dependent upon TnrA (38). The *ccpA* mutant strain grows more slowly than the wild-type strain in glucose minimal medium containing glutamine (Table 5). Since *nrgAB* is expressed at low levels in both wild-type and *ccpA* mutant cells grown in glucose glutamine minimal medium (Table 5), the growth defect seen in the *ccpA* mutant in this medium does not result from constitutive expression of TnrA-regulated genes. Surprisingly, although wild-type and *ccpA* cultures exhibited similar growth rates under nitrogen-limited conditions (i.e., glucose glutamate minimal medium), *nrgAB* expression increased almost 10,000-fold in the wild-type strain but only 12-fold in the *ccpA* mutant (Table 5).

The pattern of *nrgAB* expression in the *ccpA* mutant is similar to that previously seen in a *B. subtilis ptsI* mutant (41). The *ptsI* gene encodes the enzyme I component of the PTS and is required for transport of all PTS-dependent carbohydrates (29). Since glucose transport is deficient in *ptsI* mutants, the

TABLE 5. β-Galactosidase expression of an *nrgAB-lacZ* fusion in wild-type and *ccpA* mutant strains

Relevant genotype[a]	Carbon source(s)	Value for cells grown with nitrogen source[b]:			
		Glutamine		Glutamate	
		Doubling time (min)	β-Galactosidase sp act (U/mg of protein)	Doubling time (min)	β-Galactosidase sp act (U/mg of protein)
Wild type	Glucose	53	0.04	165	288
	Glucose + lactate	55	0.06	180	310
ccpA	Glucose	86	0.04	155	0.5
	Glucose + lactate	85	0.05	195	399

[a] All strains were derivatives of 168 and contained the (*nrgA-lacZ*)*407* fusion.
[b] β-Galactosidase levels are the averages of three or more determinations, which did not vary by more than 25%. Cultures were grown in MOPS minimal medium containing the indicated carbon and nitrogen sources.

ptsI mutant grows at a reduced rate in glucose minimal medium. The expression of the TnrA-dependent *nrgAB* promoter is not activated in a *ptsI* mutant grown in glucose minimal medium containing the limiting nitrogen source glutamate (41). One explanation for this result is that because TnrA regulation occurs only during nitrogen limitation, cells whose growth is carbon limited are unable to sufficiently deplete intracellular nitrogen pools to allow high-level expression of TnrA-regulated genes. If reduced intracellular carbon pools are responsible for the inability to completely derepress *nrgAB* expression in the *ccpA* mutant, high-level *nrgAB* expression might be observed in *ccpA* cultures grown with glucose and additional carbon sources. Indeed, *nrgAB* was expressed at similar levels in wild-type and *ccpA* mutant strains grown in minimal medium with a limiting nitrogen source (glutamate) and either glucose plus lactate or glucose plus citrate as carbon sources (Table 5 and data not shown). This suggests that the *ccpA* mutant may contain lower levels of intracellular tricarboxylic acid cycle intermediates than wild-type cells. However, because neither lactate nor citrate was able to suppress the growth phenotype of the *ccpA* mutant in glucose-plus-glutamine minimal medium (Table 5 and data not shown) or restore CCR of *hut* expression (data not shown), additional metabolic perturbations must be present in the *ccpA* mutant.

Role of glucose kinase in CCR of *hut* expression. Glc-6-P has been proposed to be involved in CCR of the *B. subtilis gnt* and *B. megaterium xyl* operons (16, 22). To determine whether Glc-6-P is required for wild-type levels of CCR of *hut* expression, expression of the HUT646 (*hut-lacZ*) fusion was examined in wild-type and glucose kinase (*glcK*) mutant cultures. The observation that β-galactosidase is expressed from the HUT646 fusion at similar levels in wild-type and *glcK* strains (Table 2) indicates that glucose kinase most likely does not participate in CCR of *hut* expression in *B. subtilis*.

The experimental evidence that Glc-6-P functions as an in vivo signal for carbon availability is equivocal for several reasons. First, the Glc-6-P-dependent in vitro binding of *B. megaterium* CcpA to *xyl cre* (16) was observed only with acidic conditions (pH 4.1 to 5.4) that differ significantly from the experimentally observed cytoplasmic pH of *B. megaterium* (20). Second, the concentration of Glc-6-P used to demonstrate in vitro binding of *B. subtilis* CcpA to *gnt cre* (30 mM) (22) is significantly higher than the in vivo concentration measured in glucose-grown *B. subtilis* cells (1.35 to 4.4 mM) (7, 10). Finally, since the intracellular concentrations of Glc-6-P are similar in glucose-grown *B. subtilis* and *E. coli* cells (25), the observation that CcpA is unable to mediate CCR regulation of *gnt* expression in *E. coli* cells (23) argues that Glc-6-P does not function as an in vivo corepressor for CcpA.

Role of CcpB in CCR in *hut* expression. CcpB, a homolog of the CcpA protein, has been reported to be involved in CCR of *gnt* and *xyl* expression in cells grown in liquid cultures with low levels of aeration or on solid medium (3). No differences in histidase expression from the *hut* operon were seen in the wild-type and *ccpB* mutant strains grown in minimal medium (data not shown). Furthermore, as judged by colony color, no difference in β-galactosidase expression from the HUT924 *lacZ* fusion was observed in wild-type and *ccpB* cells grown on X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) plates containing either LB glucose medium or glucose glutamine minimal medium (data not shown). Thus, there is no evidence that CcpB participates in CCR of *hut* expression.

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