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Regulation of Histidine and Proline Degradation Enzymes by Amino Acid Availability in *Bacillus subtilis*

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The first enzymes of the histidine (*hut*) and proline degradative pathways, histidase and proline oxidase, could not be induced in *Bacillus subtilis* cells growing in glucose minimal medium containing a mixture of 16 amino acids. Addition of the 16-amino-acid mixture to induced wild-type cells growing in citrate minimal medium repressed histidase synthesis 25- to 250-fold and proline oxidase synthesis 16-fold. A strain containing a transcriptional fusion of the *hut* promoter to the β-galactosidase gene was isolated from a library of Tn*917-lacZ* transpositions. Examination of histidase and β-galactosidase expression in extracts of a *hut-lacZ* fusion strain grown in various media showed that induction, catabolite repression, and amino acid repression of the *hut* operon were mediated at the level of transcription. This result was confirmed by measurement of the steady-state level of *hut* RNA in cells grown in various media. Since amino acid repression was not defective in *B. subtilis* mutants deficient in nitrogen regulation of glutamine synthetase and catabolite repression, amino acid repression appears to be mediated by a system that functions independently of these regulatory systems.

In *Bacillus subtilis*, four enzymes are responsible for the degradation of L-histidine to ammonium (NH_4^+), L-glutamate, and formamide (19). Genetic and biochemical analysis of the histidine utilization (*hut*) genes showed linkage to a locus that regulates their expression (4, 16). This finding suggested that the *hut* genes are likely to be organized as an operon.

The *hut* mutations define the map order *hut(R,P)CHUIG* (4, 16). The *hutH*, *hutU*, *hutI*, and *hutG* genes encode histidase, urocanase, imidazolone propionate aminohydrolase, and formimino-L-glutamate formiminohydrolase, respectively. Expression of the *hut* enzymes does not occur in strains containing the *hutP* mutation, whereas the *hutC* mutation results in constitutive synthesis of the *hut* enzymes (4). Synthesis of the *hut* enzymes is insensitive to catabolite repression (CR) in *hutR* strains (4).

B. subtilis DNA encoding the *hut* regulatory region, the *hutH* gene, and part of the *hutU* gene has been cloned and sequenced (23). The *hutP* mutation lies within the first open reading frame in the *hut* operon, and the HutP protein may positively regulate *hut* expression. Since the noncoding region between the *hutP* and *hutH* genes contains a sequence that could form a stem-loop structure, antitermination of transcription has been proposed to be involved in induction of the *hut* operon by histidine. Primer extension analysis has shown that the *hutP*, *hutH*, and *hutU* genes are transcribed from a single promoter (23).

Synthesis of the *hut* enzymes is regulated by histidine induction and CR (4, 14, 16). Since histidase expression is elevated during vegetative growth in early-blocked sporulation mutants (*spo0*), the *hut* genes have also been proposed to be regulated by *spo0* gene products (2, 3).

The enzymes involved in proline degradation in *B. subtilis* have not been extensively characterized, and mutants deficient in proline utilization (*put*) have not been reported. Proline oxidase, the first enzyme in proline degradation, is induced by proline in *B. subtilis*; its synthesis is not significantly regulated by CR (9). Proline is apparently degraded to glutamate in *B. subtilis*, since proline serves as a source of glutamate for *B. subtilis* mutants deficient in glutamate synthase (S. Fisher, unpublished observations).

The *hut* enzymes of both *Klebsiella aerogenes* and *B. subtilis* are inducible, and their synthesis can be activated during growth on a poor carbon source (4, 20). Synthesis of these enzymes can also be derepressed by NH_4^+ limitation in *K. aerogenes* but not in *B. subtilis* (1, 4, 20, 30, 32). Derepression of *hut* expression by NH_4^+-limited growth in *K. aerogenes* requires both the *nac* and *ntrC* gene products (1, 20). The *ntrC* gene is a member of the global Ntr (nitrogen regulation) system which positively regulates the expression of glutamine synthetase (GS), amino acid permeases, and NH_4^+-generating degradative enzymes during NH_4^+ limitation in enteric bacteria (27). Although expression of the *hut* operon is not altered by NH_4^+ limitation in *B. subtilis*, the expression of several other nitrogen-degradative enzymes is activated during NH_4^+-limited growth in *Bacillus* species, e.g., urease in *B. subtilis* and asparaginase in *B. subtilis* and *Bacillus licheniformis* (12; M. Atkinson and S. Fisher, unpublished observations).

Investigations of the regulation of gene expression in response to NH_4^+ availability in *B. subtilis* have shown that this regulation differs considerably from that seen in enteric bacteria. GS synthesis is negatively regulated by the *glnR* gene product, which is a member of the *glnRA* operon (29). There is no evidence that the *glnR* protein participates in a global nitrogen regulatory system in *B. subtilis*. Hence, we considered the possibility that the *hut* operon is nitrogen regulated in *B. subtilis* but that this regulation is mediated by different signals than in enteric bacteria. We report here that expression of the *hut* and *put* enzymes in *B. subtilis* can be repressed by growth in the presence of amino acids and that this regulation appears to be distinct from the system that regulates GS expression in *B. subtilis*.

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TABLE 1. *B. subtilis* strains and plasmids

Strain or plasmid	Description[a]	Source or reference
Strains		
SH	Wild type	B. Magasanik (4)
SH32	*hutC1*	B. Magasanik (4)
SH3	*hutH1*	B. Magasanik (4)
SF4	*spo0B6Z gltA292 hutR4*	7
SF34	*hutR4*	This work, transformation of SH3 with SF4 DNA
SF321	*metC3 trpC2 hutC1*	7
SF331	*metC3 hutC1 cdh-3*	7
SF10	Wild type	9
SF22	*glnA22*	9
SF73	*glnA73*	9
SB12	*glnR1*	29
HJS31	*glnR57*	29
168	*trpC2*	This laboratory
PY350	*trpC2 thrA5*	P. Youngman
SF5	*trpC2 thrA5 hutU*::Tn*917-lacZ*	This work, Tn*917-lacZ* mutagenesis
SF1685	*trpC2 hutU*::Tn*917-lacZ*	This work, transformation of 168 with SF5 DNA
SF1686	*hutU*::Tn*917-lacZ*::pTV21Δ2 *trpC2*	This work, transformation of SF1685 with *Xba*I-digested pTV21Δ2 DNA
Plasmids		
pTV32Ts	Cm^r Erm^r	P. Youngman (35)
pTV21Δ2	Amp^r Cm^r	P. Youngman (35)
pSFB1	Amp^r Cm^r	This work, derived from SF1686 by digestion of chromosomal DNA with *Bgl*II, dilute ligation, and transformation into HB101; contains 1.32 kb of *hutHU* DNA (Fig. 1)

[a] *hutH*, Loss of histidase activity; *hutU*, loss of urocanase activity; *hutC*, constitutive synthesis of *hut*; *hutR*, resistant synthesis of histidine-degrading enzymes to CR; *cdh-3*, catabolite-insensitive *hut* expression: Amp^r, ampicillin resistance; Erm^r, erythromycin resistance; Cm^r, chloramphenicol resistance.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used are listed in Table 1. *B. subtilis* chromosomal DNA for transformations and competent cells of *B. subtilis* were prepared as previously described (33). The *hut* phenotype was scored on citrate-histidine minimal plates. The *Escherichia coli* cloning host was HB101, and competent *E. coli* cells were prepared as described by Maniatis et al. (21).

Media and cell growth. Tryptose blood agar plates and Difco sporulation medium have been previously described (33). The basal salts solution (BSS) for minimal medium is described elsewhere (4). The carbon source was 0.5% glucose, 0.2% inositol, 0.2% maltose, or 0.2% sodium citrate. The final concentration of all nitrogen sources was 0.2%. When L-glutamate was the nitrogen source, 150 mM NaCl was added to facilitate glutamate transport. L-Histidine, L-proline, and L-arginine hydrochloride were added at 0.1% to induce their degradative enzymes. L-Glutamine and L-histidine were freshly prepared for each experiment and filter sterilized.

The 16-amino-acid medium contained (in milligrams per liter of BSS solution) L-cystine (40), L-arginine hydrochloride (400), L-isoleucine (200), L-leucine (200), L-valine (200), L-glutamate (800), L-lysine (100) L-phenylalanine (100), L-proline (100), L-threonine (100), L-aspartate (665), L-alanine (445), glycine (375), L-serine (525), L-tryptophan (150), and L-methionine (160) and was sterilized by autoclaving. The amino acids cystine, lysine, phenylalanine, proline, and threonine were omitted in the 11-amino-acid medium. The five-amino-acid mixture contained isoleucine, leucine, valine, tryptophan, and methionine at the concentrations given above. The six-amino-acid medium contained arginine, glutamate, aspartate, alanine, glycine, and serine at the concentrations given above.

Cell growth was monitored with a Klett-Summerson colorimeter (green filter). Overnight BSS minimal cultures were prepared by inoculating several cultures at various cell densities with cells in exponential growth on a tryptose blood agar plate. The next day, an exponential overnight culture was diluted into BSS medium so that the turbidity of the culture was 5 Klett units.

Isolation of *hutU*::Tn*917-lacZ* fusion. A Tn*917-lacZ* insertion in the *hut* operon was isolated by screening a library of Tn*917-lacZ* insertions for Hut^- colonies that produced β-galactosidase only in medium containing histidine. This library was generated by using plasmid pTV32Ts (35). One Tn*917-lacZ hut* insertion, SF5, was isolated after screening 4,000 colonies. When the Tn*917-lacZ* insertion from strain SF5 was transformed into strain 168, the Hut^- phenotype cotransformed 100% with erthromycin resistance. Extracts of one of these transformants, SF1685, contained wild-type levels of histidase activity but no urocanase activity (data not shown).

To verify that the Tn*917-lacZ* insertion had occurred within the *hut* operon, chromosomal DNA adjacent to the transposon arm containing the promoterless *lacZ* gene was cloned in *E. coli* by using the integration vector pTV21Δ2 (Fig. 1) as described previously (35). The resulting plasmid, pSFB1, contained 1.32 kilobase pairs (kb) of *B. subtilis* chromosomal DNA and could transform the *hutH1* strain, SH3, to Hut^+. Restriction site mapping of the *B. subtilis* chromosomal DNA in pSFB1 showed that the Tn*917-lacZ* insertion occurred 220 base pairs (bp) downstream of the *Nde*I site that partially overlaps the *hutU* ATG start codon (23; Fig. 1 and data not shown).

Preparation of cell extracts and enzyme assays. Cells (50 ml) were grown to mid-log growth phase (70 to 90 Klett units), harvested by centrifugation for 10 min in a Sorvall SS-34 rotor, washed with 25 ml of the appropriate buffer, and stored at −20 or −70°C. Cell extracts were prepared by resuspending thawed cells in 1 ml of the appropriate buffer and incubating the cells with 200 μg of lysozyme per ml for

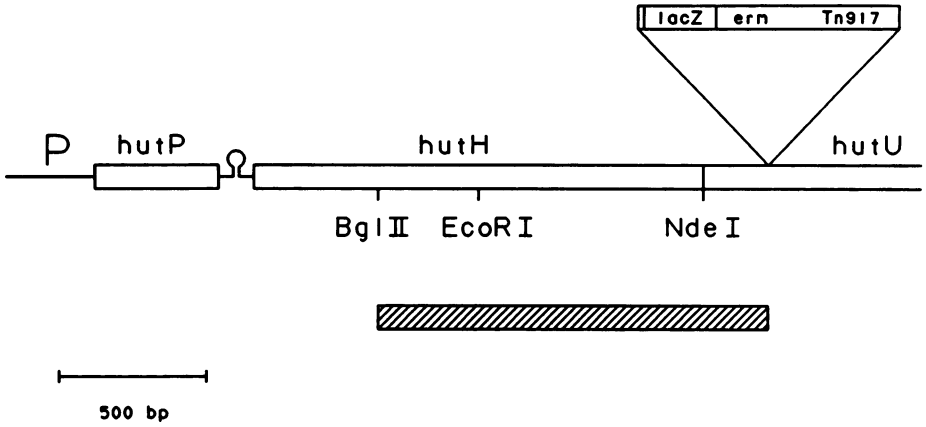

FIG. 1. The *hutU*::Tn*917-lacZ* insertion in the *hut* operon. The restriction and physical map of the *hutPHU* genes is taken from Oda et al. (23). ▨, The cloned *hutHU* DNA fragment used as the DNA probe in the RNA slot blots. The Tn*917-lacZ* transposon is not drawn to scale. Abbreviations: P, *hut* promoter region; *hutP*, *hut* regulatory protein; *hutH*, histidase structural gene; *hutU*, urocanase structural gene; *erm*, erythromycin resistance gene; *lacZ*, promoterless β-galactosidase gene.

5 to 10 min at 37°C. After lysis, the cell extract was cooled to 4°C and briefly sonicated to reduce viscosity. Alternately, the cells were incubated with lysozyme and 50 μg of DNase per ml. Cell debris was removed by centrifugation, and the supernatant was used for enzyme assays.

Cells for histidase assays were washed and lysed in 50 mM potassium phosphate buffer (pH 7.5). Histidase was assayed as described previously (14). One unit of histidase activity produced 1 nmol of urocanic acid per min. Extracts for the urocanase assay were prepared with 20 mM Tris (pH 8) and 35 μg of pyridoxal phosphate per ml. After sonication, streptomycin was added to the cell extract at a final concentration of 1%. This mixture was incubated at 4°C for 30 min and centrifuged, and the supernatant was used to assay urocanase as described previously (4). One unit of urocanase activity destroyed 1 nmol of urocanic acid per min.

The buffer for preparation of cell extracts for aconitase, inositol dehydrogenase, and α-glucosidase assays was 50 mM potassium phosphate buffer (pH 7.5) containing 150 mM $NaCl_2$. Aconitase was assayed by the method of Cox and Hanson (5). One unit of aconitase activity produced 1 nmol of *cis*-aconitate per min. Inositol dehydrogenase levels were determined by the method of Levin and Magasanik (17). One unit of inositol dehydrogenase activity caused the reduction of 1 nmol of NAD^+ per min. α-Glucosidase was assayed as described previously (4).

β-Galactosidase was measured in cells frozen in liquid nitrogen immediately after harvesting and stored at −70°C. Cell extracts were prepared in Z buffer (22). β-Galactosidase was assayed by adding up to 0.2 ml of cell extract to a test tube containing sufficient Z buffer to bring the final volume to 1 ml. After 10 min of incubation at 28°C, 0.2 ml of a 4-mg/ml solution of *o*-nitrophenyl-β-D-galactoside was added. When sufficient yellow color had developed, the assay was stopped with 0.5 ml of 1 M Na_2CO_3 and the incubation time was noted. The assay mixture was centrifuged, and the A_{420} of the supernatant was determined. One unit of β-galactosidase activity produced 1 nmol of *o*-nitrophenol per min. β-Galactosidase specific activity was always corrected for endogenous *B. subtilis* β-galactosidase activity, e.g., 0.13 in glucose-grown cells and in 1.2 in citrate-grown cells.

Proline oxidase was measured as described previously (9). One unit of proline oxidase activity produced 1 nmol of *o*-aminobenzaldehyde per min. Arginase was assayed by the method of Harwood and Baumberg (15). GS activity was determined with the Mn^{2+}-dependent reverse transferase assay in permeabilized cells (9). One unit of GS activity produced 1 nmol of α-glutamylhydroxamate per min.

Protein concentration was determined by the method of Lowry et al. (18), using bovine serum albumin as a standard.

RNA slot blots. RNA was located from *B. subtilis* cells by using guanidine thiocyanate and $CsCl_2$ centrifugation as described previously (10) except that the cells were disrupted in a French pressure cell at 16,000 to 18,000 lb/in^2. The RNA samples were treated with RNase-free DNase (Promega Biotec) as described by the supplier. RNA samples (30 μl) were loaded into adjacent wells in a Schleicher & Schuell MinifoldII apparatus and dried with a heat lamp, and the nitrocellullose was baked for 1 h at 75°C. The DNA probe for the RNA dot blot, the 1.32-kb *AvaI-BglII* DNA fragment from pSFB1 (Fig. 1), was eluted from an agarose gel and nick translated. Since *AvaI* cuts 6 bp from the end of the transposon (25), the DNA probe contained primarily *B. subtilis* chromosomal DNA. Hybridization and blot washes were performed at 58.5°C as described by Maniatis et al. (21) except that the hybridization solution contained 5× SSPE, 5× Denhardt reagent, 200 μg of denatured salmon sperm DNA per ml, and 0.1% sodium dodecyl sulfate, and the final wash (0.1× SSPE–0.1% sodium dodecyl sulfate) was at 60°C.

RESULTS

Amino acid regulation of *hut*. To determine whether expression of the *hut* operon was altered by nitrogen availability, histidase levels were assayed in extracts of *B. subtilis* 168 cells grown in minimal medium containing various nitrogen and carbon sources. GS was also measured in these cultures to see whether changes in histidase levels could be correlated with GS levels. In general, histidase levels were reduced in cells grown in medium containing nitrogen sources that resulted in faster growth rates (Table 2). However, histidase expression could not always be strictly correlated with the growth rate of the culture. This was most obvious when the nitrogen source of the medium was NH_4^+ plus 5 or 11 amino acids (Table 2, lines 7 and 10).

GS levels were not always reduced in cultures with repressed histidase expression (Table 2). Addition of a

TABLE 2. Histidase and glutamine synthetase specific activities in strain 168 cells grown with different nitrogen and carbon sources[a]

Carbon source	Nitrogen source[b]	Doubling time (min)	Sp act (U/mg of protein)[c]	
			GS	Histidase
1. Glucose	N	90	ND[d]	<0.5
2. Glucose	Glt + His	135	1,071	30
3. Glucose	His	700	1,154	27
4. Glucose	N + His	90	254	11
5. Glucose	N + Glt + His	85	175	8.5
6. Glucose	Gln + His	70	139	4.2
7. Glucose	N + 5 aa + His	115	238	4.1
8. Glucose	6 aa + His	65	214	3.0
9. Glucose	N + Glt + Gln + Arg + His	55	79	2.5
10. Glucose	11 aa + His	70	245	<0.5
11. Glucose	N + 16 aa + His	45	223	<0.5
12. Glucose	16 aa + His	45	194	<0.5
13. Glucose	16 aa + Gln + His	45	103	<0.5
14. Citrate	Gln + His	110	139	203
15. Citrate	16 aa + Gln + His	45	103	0.8

[a] Cells were grown in BSS minimal medium containing the indicated nitrogen and carbon sources.
[b] His, 0.1% L-histidine added to induce the *hut* enzymes; N, NH_4^+; Gln, L-glutamine; Glt, L-glutamate; Arg, arginine; aa, amino acid mixture (see Materials and Methods).
[c] Values are averages of two to four determinations which varied by 20% or less.
[d] ND, Not determined.

mixture of 16 amino acids to induced cells growing in medium containing either NH_4^+ or glutamine as the nitrogen source severely repressed the expression of histidase but not of GS, although amino acid-containing cultures grew faster than cultures lacking amino acids (Table 2; compare lines 4 and 11, 6 and 13, and 14 and 15). On the other hand, the expression of both GS and histidase was reduced in cells grown in medium containing high concentrations of amino acids that are metabolized to glutamate as a nitrogen source. GS and histidase levels were lower in extracts of induced cells grown either with L-glutamine or with nitrogen, L-glutamate, L-glutamine, and arginine as the nitrogen sources than in NH_4^+-grown cells (Table 2, lines 4, 6, and 9). As has been previously described (24, 26, 31, 32), the expression of GS but not of histidase was derepressed to high levels in induced cells grown in medium containing poor nitrogen sources such as histidine and glutamate (Table 2, lines 2 to 4).

Repression of histidase synthesis by various mixtures of amino acids. To determine whether all 16 amino acids in the amino acid mixture were required for histidase repression, histidase levels were determined in extracts of strain 168 cells grown in glucose minimal medium containing various combinations of these amino acids. Histidase activity was not detected in extracts of induced 168 cells grown in medium containing a mixture of 11 amino acids as the nitrogen source (Table 2, line 10). The 11-amino-acid mixture was subdivided into two groups, the 5-amino-acid mixture (isoleucine, leucine, valine, tryptophan, and methionine) and the 6-amino-acid mixture (alanine, glycine, serine, arginine, glutamate, and aspartate). Histidase levels in extracts of induced cells grown with five amino acids plus NH_4^+ were 2.7-fold lower than in extracts of NH_4^+-grown cells (Table 2, lines 4 and 7). When the six-amino-acid mixture was the nitrogen source of the medium, histidase levels were 3.6-fold lower than the levels seen in NH_4^+-grown cells (Table 2, lines 4 and 8). Examination of histidase levels in cultures grown in medium containing various combinations of the amino acids in the 6-amino-acid mixture showed that histidase repression could not be attributed to any amino acid subset (data not shown).

TABLE 3. Proline oxidase specific activity in strain 168 cells grown with different nitrogen and carbon sources[a]

Carbon source	Nitrogen source[b]	Doubling time (min)	Proline oxidase act (U/mg of protein)[c]
Glucose	N	85	<0.5
Glucose	N + Pro	75	10
Glucose	16 aa + N + Pro	55	<1
Citrate	Gln + Pro	115	16
Citrate	16 aa + Gln + Pro	55	1

[a] Cells were grown in BSS minimal medium containing, the indicated nitrogen and carbon sources.
[b] Pro, 0.1% L-proline added to induce proline-degrading enzymes, N, NH_4^+; Gln, glutamine; aa, amino acid mixture (see Materials and Methods).
[c] Values are averages of two to three determinations which varied by 20% or less.

Amino acid regulation of the proline and arginine degradative enzymes. Proline oxidase levels were reduced 10- and 16-fold, respectively, when the 16-amino-acid mixture was added to induced 168 cells growing in minimal medium containing either glucose or citrate as the carbon source (Table 3). In contrast, addition of amino acids to the growth medium repressed the expression of arginase, the first enzyme of the arginine degradative pathway, only twofold in induced cells in glucose minimal medium compared with the levels seen in cells growing in the same medium but lacking amino acids (data not shown).

The *hut* operon is regulated at the transcriptional level. Strain SF1685 (*hutU*::Tn*917-lacZ*) contains a *lacZ* transcriptional gene fusion to the *hut* promoter. To investigate regulation of the *hut* operon, histidase and β-galactosidase activities were determined in extracts of SF1685 cells grown in various media. Since histidase and β-galactosidase activities varied in parallel in SF1685 cells grown in various growth media (data not shown), induction, catabolite repression, and amino acid repression of the *hut* operon are mediated transcriptionally.

This observation was verified by using RNA slot blots to quantitate *hutHU* RNA present in RNA isolated from 168 cells grown in various media. No *hutHU* RNA could be detected in RNA isolated either from uninduced cells or from induced cells grown in medium containing glucose and the 16-amino-acid mixture (Fig. 2, bands 1 and 3). Densitometric scanning of the autoradiograph showed that *hutHU* RNA levels in RNA extracted from induced glucose-grown cells were 15.7-fold lower than they were in RNA isolated from induced citrate-grown cells (Fig. 2, bands 2 and 4). This finding correlates well with histidase activity, which was 17.7-fold lower in glucose-grown cells than it was in citrate-grown cells. The levels of histidase and *hutHU* RNA were 13- and 19.7-fold lower, respectively, in induced cells grown in medium containing citrate, glutamine, and 16 amino acids than they were in cells grown in the same medium without amino acids (Fig. 2, bands 4 and 5).

Amino acid repression in the *hutC* mutant. If histidine transport were inhibited in cells growing in the presence of amino acids, then amino acid repression of *hut* expression could be due to inducer exclusion. This possibility was tested by determining whether histidase expression is repressed by amino acids in strain SH32, which synthesizes

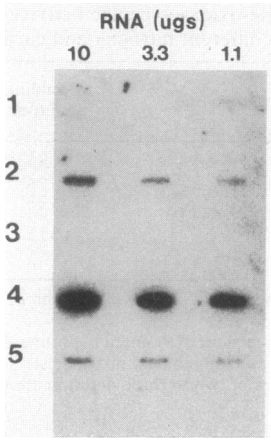

FIG. 2. Slot blot analysis of *hutHU* RNA. RNA was isolated from cells grown in various media and treated with DNase before threefold dilutions of each RNA were bound to a nitrocellulose filter. The blot was hybridized with the 1.32-kb *Bgl*II-*Ava*I *hutHU* DNA fragment shown in Fig. 1. RNA samples used were isolated from exponentially growing strain 168 cells in the following media: (1) glucose–NH_4^+–glutamate; (2) glucose–NH_4^+–glutamate–histidine; (3) glucose–NH_4^+–glutamate–histidine–16 amino acids; (4) citrate–glutamine–histidine; and (5) citrate–glutamine–histidine–16 amino acids. Histidase specific activities (units per milligram of protein) measured in extracts of these cultures were <0.5 (1), 9.6 (2), <0.5 (3), 170 (4), and 13 (5).

the *hut* enzymes constitutively as a result of the *hutC1* mutation (4). Since histidase activity could not be detected in extracts of either uninduced or induced *hutC1* cells growing with NH_4^+ and 16 amino acids as the nitrogen source (Table 4), histidine transport is unlikely to be involved in the mechanism mediating amino acid repression.

Amino acid repression in *B. subtilis* mutants deficient in CR. The mechanism mediating CR of the *hut* operon is not understood, although the complete derepression of histidase expression can be correlated with reduced intracellular pools of 2-ketoglutarate and pyruvate in *B. subtilis* (8). Since the carbon backbone of many amino acids can be metabolized to 2-ketoglutarate and pyruvate, CR and amino acid repression of *hut* expression could be mediated by the same regulatory mechanism. If so, amino acid repression should be defective in *B. subtilis* mutants deficient in CR of the *hut* operon.

Histidase synthesis is not subject to regulation by CR in strain SF34, which contains the *hutR4* mutation (7; Table 4). Since the *hutR4* mutation is tightly linked to the *hutH1* mutation, this mutation is likely to lie in the *hut* promoter region (7). Similar levels of histidase activity were seen in extracts of wild-type and SF34 cells grown in medium containing amino acids (Table 4).

CR is partially relieved in strains SF22 and SF73, which contain mutations in the GS structural gene (*glnA*) (9). Histidase levels in extracts of induced SF22 and SF73 cells grown in medium containing glucose plus glutamine were 3.5- to 4-fold higher than the levels seen in extracts of wild-type cells (Table 5). However, histidase activity could not be detected in extracts of either induced wild-type or *glnA* mutant cells grown in medium containing amino acids (Table 5). Proline oxidase levels were twofold higher in extracts of glucose-grown SF22 and SF73 cells than in extracts of wild-type cells (Table 5). Proline oxidase levels were 4.5-fold lower in extracts of induced SF22 and SF73 cells grown in medium containing amino acids than in extracts of SF22 and SF73 cells grown in the same medium but without amino acids (Table 5).

The regulation of enzymes subject to CR is also partially relieved in the *cdh-3* mutant (7). Aconitase and histidase levels were 4-fold and 4.5-fold higher, respectively, in extracts of induced SF331 (*cdh-3*) cells grown with glucose as the carbon source than in extracts of wild-type cells (Table 6). The addition of amino acids to the growth medium resulted in a 6.5-fold reduction in histidase activity and a 3- to 4.5-fold reduction in aconitase activity in extracts of both wild-type and *cdh-3* mutant cells (Table 6).

The expression of other carbon degradative enzymes subject to regulation by CR was examined in extracts of cells grown with and without amino acids. Similar levels of inositol dehydrogenase were present in extracts of cells grown in medium containing inositol as the carbon source with or without amino acids (Table 7). Inositol dehydrogenase levels were 4.5-fold higher in extracts of cells grown in glucose–inositol–glutamate–16 amino acids than in extracts of cells grown in the same medium but lacking the amino acids (Table 7). Similarly, addition of amino acids to cells growing in medium containing maltose as the carbon source did not alter α-glucosidase levels in cells extracts (data not shown). In summary, these studies give no evidence that the mechanisms mediating CR and amino acid repression are at all related.

Amino acid repression in *glnR* mutants. To test whether the GlnR protein is involved in amino acid regulation, expression of the *hut* and *put* degradative enzymes was examined in strains HJS31 and SB12, which synthesize GS constitutively as a result of mutations in the *glnR* gene (29). Identical levels of histidase and proline oxidase were present in extracts of induced wild-type and *glnR* cells grown in either the presence or absence of amino acids (Table 5).

TABLE 4. Histidase specific activity in wild-type and *hut* mutants

Medium[a]	SH (wild type)		SH32 (*hutC32*)		SF34 (*hutR4*)	
	Doubling time (min)	Histidase sp act (U/mg of protein)[b]	Doubling time (min)	Histidase sp act (U/mg of protein)	Doubling time (min)	Histidase sp act (U/mg of protein)
Glucose + N	80	<0.5	83	7 ± 1	83	2 ± 0.2
Glucose + N + His	80	9 ± 3	80	28 ± 5	85	338 ± 35
Glucose + N + 16 aa	ND[c]	ND	45	<0.5	ND	ND
Glucose + N 16 aa + His	45	<0.5	48	<0.5	45	<0.5
Citrate + Gln + His	100	293 ± 77	ND	ND	105	432 ± 68
Citrate + His + Gln + 16 aa	70	3 ± 4	ND	ND	67	5 ± 0.4

[a] Cells were grown in BSS minimal medium containing the indicated nitrogen and carbon sources. For abbreviations, see Table 2, footnote *b*.
[b] Values are averages of two to four determinations. The error is the standard deviation.
[c] ND, Not determined.

TABLE 5. Histidase and proline oxidase activities in wild-type cells, GS structural mutants, and GS regulatory mutants

Strain	Relevant genotype	Nitrogen source[a]	Histidase		Proline oxidase	
			Doubling time (min)	Sp act (U/mg of protein)	Doubling time (min)	Sp act (U/mg of protein)
SF10	Wild type	N + Glt	75	12	75	5.5
		Gln	60	6	ND[c]	ND
		16 aa + N + Glt	45	<0.6	45	<1.3
SF22	*glnA22*	Gln	100	47	90	12.9
		16 aa + Gln	75	<0.6	65	2.6
SF73	*glnA73*	Gln	90	41	85	11.9
		16 aa + Gln	65	<1	55	2.7
HJS31	*glnR57*	N + Glt	65	16	55	6.2
		16 aa + N + Glt	45	<0.5	40	<1.6
SB12	*glnR1*	N + Glt	70	13	65	6.9
		16 aa + N + Glt	48	<0.7	48	<1.1

[a] Cells were grown in BSS minimal medium containing 0.5% glucose, the indicated nitrogen sources, and either 0.1% L-histidine or L-proline to induce histidase and proline oxidase, respectively. For abbreviations, see Table 2, footnote *b*.
[b] Values are averages of two independent determinations which varied by 20% or less.
[c] ND, Not determined.

DISCUSSION

We have shown that the *B. subtilis hut* and *put* enzymes are subject to a novel form of regulation. The expression of these amino acid-degrading enzymes is severely repressed by the addition of a mixture of 16 amino acids to cells growing in minimal medium containing either glucose or citrate as the carbon source. Repression of histidase synthesis by the 16-amino-acid mixture could not be fully attributed to any one subset of amino acids in the 16-amino-acid mixture. This finding suggests that amino acid repression is mediated in response to the general availability of amino acids rather than to the availability of a single amino acid such as glutamate. Indeed, cells growing in glucose minimal medium containing the mixture of 16 amino acids grew faster than on other nitrogen sources.

If amino acid repression is mediated in response to nitrogen availability, then the mechanism mediating this regulation might overlap with the system mediating nitrogen regulation of GS. This would not be unprecedented, because in enteric bacteria the Ntr system regulates the expression of both GS and nitrogen-degradative enzymes in response to NH_4^+ availability. However, the addition of amino acids to the growth medium repressed the expression of histidase but not of GS. Furthermore, amino acid repression was not deficient in *B. subtilis* strains that synthesize GS constitutively as a result of mutations either in the *glnR* gene (29) or in the *glnA* gene (6, 31). All of these data suggest that the system mediating amino acid repression of the *hut* and *put* enzymes functions independently of the mechanism regulating GS expression in *B. subtilis*.

In *E. coli*, the addition of rapidly catabolized amino acids such as serine to a glucose-containing medium results in a twofold reduction in β-galactosidase synthesis (34). This raises the possibility that amino acid repression and CR are mediated by the same mechanism in *B. subtilis* and that amino acid repression results from the enhancement of CR in *B. subtilis*. Two lines of evidence support the hypothesis that amino acid repression and CR are mediated by independent mechanisms: (i) the expression of two carbon-degradative enzymes subject to regulation by catabolite repression was not repressed by addition of amino acids to the growth medium, and (ii) amino acid repression of histidase and proline oxidase expression was not defective in *B. subtilis* mutants generally deficient in regulation by CR or in a mutant specifically defective in CR of *hut*. However, since the mechanisms involved in CR in *B. subtilis* have not been identified (32), and since the defect in CR caused by the *hutR*, *glnA*, and *cdh-3* mutations are not completely understood, we cannot definitively rule out the possibility that the mechanisms mediating CR and amino acid repression are interrelated.

In contrast to the results seen with inositol dehydrogenase and α-glucosidase, the synthesis of aconitase was repressed 3- to 4-fold by addition of the 16-amino-acid mixture to cells growing in medium containing glucose, glutamate, and NH_4^+. Aconitase expression has been shown to be re-

TABLE 6. Histidase and aconitase activities in wild-type and *cdh-3* mutants

Strain	Relevant genotype	Nitrogen source[a]	Doubling time (min)	Sp act (U/mg of protein)[b]	
				Histidase	Aconitase
SF321	*hutC1*	N + Glt	65	10	9
		16 aa + N + Glt	50	1.5	3
SF331	*hutC1 cdh-3*	N + Glt	65	46	36
		16 aa + N + Glt	45	7	8

[a] Cells were grown in BSS minimal medium containing 0.5% glucose, the indicated nitrogen sources, and 0.1% L-histidine to induce the histidine-degrading enzymes. For abbreviations, see Table 2, footnote *b*.
[b] Values are averages of two independent determinations which varied by 20% or less.

TABLE 7. Inositol dehydrogenase specific activity in strain 168 cells[a]

Carbon source	Nitrogen source	Doubling time (min)	Inositol dehydrogenase sp act (U/mg of protein)[b]
Inositol	N + Glt	115	1,083
Inositol	N + Glt + 16 aa	62	1,109
Inositol + glucose	N + Glt	60	6
Inositol + glucose	N + Glt + 16 aa	55	26

[a] Cells were grown in BSS minimal medium containing the indicated nitrogen and carbon sources. For abbreviations, see Table 2, footnote *b*.
[b] Values are averages of two independent determinations which varied by 20% or less.

pressed severalfold by growth in the presence of a rapidly metabolized carbon source such as glucose, and its synthesis can be further repressed by growth in the presence of glucose and a source of glutamate in *B. subtilis* (13, 28). Thus, the reduced synthesis of aconitase in cells growing in glucose minimal medium containing amino acids may be due either to increased availability of glutamate or to amino acid repression.

Regulation of *hut* expression by histidine induction, CR, and amino acid repression appears to be mediated at the transcriptional level in *B. subtilis*. Similar levels of histidase and β-galactosidase were present in extracts of SF1685 (*hutU*::Tn*917-lac*) cells grown in various media. The level of *hutHU* RNA present in wild-type cells grown in various media correlated well with histidase activity present in extracts of the cultures from which the RNA was isolated.

In *B. subtilis*, regulation of the *hut* and *put* enzymes by amino acids results in repression of their synthesis during vegetative growth in medium containing amino acids. At the end of exponential growth in amino acid-containing medium, the expression of both histidase and proline oxidase is activated (Atkinson and Fisher, unpublished observations). This regulation differs considerably from the situation in *K. areogenes*, in which the derepression of *hut* synthesis during NH_4^+ limitation allows this bacterium to grow rapidly in medium containing histidine as the sole nitrogen source. Amino acid repression of the *B. subtilis hut* and *put* enzymes may reflect the fact that *B. subtilis* can initiate sporulation during nutrient-limited growth. Since sporulation occurs at high frequencies during nutrient limitation but not during nutrient starvation (11), the regulation of metabolism in *B. subtilis* may have evolved so that not all of the available nutrients are consumed during rapid growth. This would ensure that adequate supplies of nitrogen, carbon, and phosphate compounds are present during sporulation to allow completion of this developmental process.

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