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Role of CodY in Regulation of the *Bacillus subtilis hut* Operon

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Bacillus subtilis **mutants deficient in amino acid repression of the histidine utilization (*hut*) operon were isolated by transposon mutagenesis. Genetic characterization of these mutants indicated that they most likely contained transposon insertions within the *codVWXY* operon. The *codY* gene is required for nutritional regulation of the dipeptide permease (*dpp*) operon. An examination of *hut* expression in a Δ*codY* mutant demonstrated that amino acid repression exerted at the $hutO_A$ operator, which lies immediately downstream of the *hut* promoter, was defective in a Δ*codY* mutant. The *codY* gene product was not required for amino acid regulation of either *hut* induction or the expression of proline oxidase, the first enzyme in proline degradation. This indicates that more than one mechanism of amino acid repression is present in *B. subtilis*. An examination of *dpp* and *hut* expression in cells during exponential growth in various media revealed that the level of CodY-dependent regulation appeared to be related to the growth rate of the culture.**

In *Bacillus subtilis*, L-histidine is degraded to ammonia, glutamate, and formamide by four enzymes, histidase, urocanase, imidazolone propionate aminohydrolase, and formimino-L-glutamate formiminohydrolase (16). These enzymes are encoded by genes that lie within the multicistronic *hut* operon (4, 13). DNA sequence analysis has revealed that the *hut* operon contains six open reading frames (19, 29). The first structural gene within the *hut* operon, *hutP*, encodes a positive regulatory protein (19). The four open reading frames immediately downstream of *hutP*, *hutHUIG*, encode the histidine catabolic enzymes (4, 13, 29). The derived amino acid sequence of the final *hut* gene, *hutM*, has sequence similarities with bacterial amino acid permeases and has been proposed to encode a histidine permease (29). A factor-independent transcriptional terminator lies between the *hutP* and *hutH* genes (19, 27).

Expression of the *B. subtilis hut* operon is tightly regulated by histidine induction, carbon catabolite repression, and amino acid repression (2, 4). The DNA sites involved in *hut* regulation were identified by genetic and deletion analyses. Histidine-dependent induction of the *hut* operon occurs primarily by transcriptional antitermination at the terminator located between the *hutP* and *hutH* genes (27). Carbon catabolite repression of *hut* expression is exerted at two sites within the *hut* operon (18, 28). The $hutO_{CR1}$ site lies immediately downstream of the *hut* promoter and only weakly regulates *hut* expression. The $hutO_{CR2}$ site, which is required for complete *hut* catabolite repression, is located over 200 bp downstream of the *hut* transcriptional start site. Amino acid repression blocks two different steps in *hut* expression (27). First, inhibition of the transport of L-histidine, the *hut* inducer, in amino acid-grown cultures prevents induction of the *hut* operon. Secondly, transcription from the *hut* promoter is negatively regulated in response to amino acid availability at an operator, $hutO_A$, that lies immediately downstream of the *hut* promoter. The activation of *hut* expression at the onset of stationary phase in *B. subtilis* cultures grown in nutrient sporulation medium has been proposed to result from the relief of amino acid repression (3).

The expression of proline oxidase and the dipeptide transport operon (*dpp*) is repressed by the addition of amino acids to *B. subtilis* cultures (2, 24). The *dpp* operon is also subject to regulation by glucose catabolite repression (24). The *codY* gene encodes a *trans*-acting factor required for both glucose catabolite repression and amino acid repression of *dpp* expression (23, 25). Purified CodY protein has been shown to bind to the *dpp* promoter region in vitro (22). During exponential growth in nutrient sporulation medium, expression of the *hut* operon is partially derepressed in *codY* mutants (25).

During a search for *trans*-acting factors required for amino acid repression mediated at the $hutO_A$ operator, transposon insertions in the *codVWXY* operon were isolated. We report here that CodY is required for amino acid regulation and carbon catabolite repression mediated at the *hut* promoter but that no defect in amino acid repression of *hut* induction or proline utilization was observed in *codY* mutants.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *B. subtilis* strains used in this study are listed in Table 1. Plasmid pCOD1 was constructed by cloning the *Eco*RI-*Hin*dIII fragment from pFS15 (25) into *Escherichia coli* plasmid pJDC9 (5). In plasmid pCOD2, a *Pst*I DNA fragment internal to the *codX* gene of pCOD1 was replaced with the neomycin resistance (Neo^r) gene from pBEST501 (12). *B. subtilis* SF20 (*codX*::*neo*) was constructed by transforming strain 168 to Neo^r with linearized pCOD2 DNA. *E. coli* DH12S (Life Technologies, Inc.) was used as the host for DNA cloning experiments.

Cell growth and media. The methods used for bacterial cultivation have been previously described (2). Difco sporulation medium, a nutrient sporulation medium (26), and the morpholinepropanesulfonic acid (MOPS) minimal medium of Neidhardt et al. (17) were used for growth of liquid cultures. Glucose was added at 0.5% to MOPS minimal medium. All other carbon and nitrogen sources were added at 0.2% to this minimal medium. L-Histidine and L-glutamine were freshly prepared and filter sterilized for each experiment.

The composition of the 16-amino-acid mixture used to repress *hut* expression was described previously (2). Mutants deficient in *hut* amino acid repression were isolated by using a 13-amino-acid mixture containing 0.008% L-cysteine, 0.04% L-isoleucine, 0.04% L-leucine, 0.04% L-valine, 0.02% L-lysine, 0.02% L-phenylalanine, 0.02% L-threonine, 0.084% L-alanine, 0.07% L-glycine, 0.105% L-serine, 0.03% L-tryptophan, 0.032% L-methionine, and 0.02% L-tyrosine.

Enzyme assays. Extracts for enzyme assays were prepared from cells harvested during exponential growth (70 to 90 Klett units) as previously described (2). Histidase and β-galactosidase were assayed in crude extracts as described previously (2). β-Galactosidase activity was always corrected for endogenous β-ga-

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

Strain	Genotype[a]	Reference, source, or derivation
168	*trpC2*	This laboratory
1A680	*lacA17 lacR1 trpC2*	*Bacillus* Genetic Stock Center (7)
SF168C	*trpC2 hutC1*	3
SF168C5	*trpC2 hutC1 hutU*::Tn*917-lacZ*	3
SF170C5	*lacA17 lacR1 hutC1 hutU*::Tn*917-lacZ trpC2*	28
SF1210	Δ*hutPH*::*neo trpC2*	28
SF2	*spo0B6Z gltA292*	8
PS37	*trpC2 unkU*::*spc* Δ*codY*	21
PS56	*trpC2 abrB*::*cat amyE*::[*neo* Φ(*dpp-lacZ*)]	21
PS58	*trpC2* Δ*amyE*::(*spc codY*)	21
SF20	*trpC2 codX*::*neo*	This work
SF168Y	*trpC2 unkU*::*spc* Δ*codY*	168 × PS37 DNA
SF168CY	*trpC2 hutC1 unkU*::*spc* Δ*codY*	SF168C × PS37 DNA
SF168CD	*trpC2 hutC1 amyE*::[*neo* Φ(*dpp-lacZ*)]	SF168C × PS56 DNA
SF168CDY	*trpC2 hutC1 amyE*::[*neo* Φ(*dpp-lacZ*)] *unkU*::*spc* Δ*codY*	SF168CD × PS37 DNA
SF603	*trpC2 amyE*::[*cam* Φ(*hut-lacZ*)*603*]	28
SF603C	*trpC2 hutC1 amyE*::[*cam* Φ(*hut-lacZ*)*603*]	SF168C × SF603 DNA
SF603CY	*trpC2 hutC1 amyE*::[*cam* Φ(*hut-lacZ*)*603*] *unkU*::*spc* Δ*codY*	SF603C × PS37 DNA
SF607	*trpC2 amyE*::[*cam* Φ(*hut-lacZ*)*607*]	28
SF607C	*trpC2 hutC1 amyE*::[*cam* Φ(*hut-lacZ*)*607*]	SF168C × SF607 DNA
SF607CY	*trpC2 hutC1 amyE*::[*cam* Φ(*hut-lacZ*)*607*] *unkU*::*spc* Δ*codY*	SF607C × PS37 DNA
SF620	*trpC2 amyE*::[*cam* Φ(*hut-lacZ*)*620*]	27
SF620C	*trpC2 hutC1 amyE*::[*cam* Φ(*hut-lacZ*)*620*]	SF168C × SF620 DNA
SF620CY	*trpC2 hutC1 amyE*::[*cam* Φ(*hut-lacZ*)*620*] *unkU*::*spc* Δ*codY*	SF620C × PS37 DNA
SF624	*trpC2 amyE*::[*cam* Φ(*hut-lacZ*)*624*]	27
SF624C	*trpC2 hutC1 amyE*::[*cam* Φ(*hut-lacZ*)*624*]	SF168C × SF624 DNA
SF624CY	*trpC2 hutC1 amyE*::[*cam* Φ(*hut-lacZ*)*624*] *unkU*::*spc* Δ*codY*	SF624C × PS37 DNA
SF626	*trpC2 amyE*::[*cam* Φ(*hut-lacZ*)*626*]	28[b]
SF626C	*trpC2 hutC1 amyE*::[*cam* Φ(*hut-lacZ*)*626*]	SF168C × SF626 DNA
SF626CY	*trpC2 hutC1 amyE*::[*cam* Φ(*hut-lacZ*)*626*] *unkU*::*spc* Δ*codY*	SF626C × PS37 DNA
SF21	*spo0B6Z gltA292 adh1*::Tn*10*	Tn*10* mutagenesis of SF2
SF25	*spo0B6Z gltA292 adh5*::Tn*10*	Tn*10* mutagenesis of SF2
SF27	*spo0B6Z gltA292 adh7*::Tn*10*	Tn*10* mutagenesis of SF2
SF210	*spo0B6Z gltA292 adh10*::Tn*10*	Tn*10* mutagenesis of SF2
SF214	*spo0B6Z gltA292 adh14*::Tn*10*	Tn*10* mutagenesis of SF2
SF217	*spo0B6Z gltA292 adh17*::Tn*10*	Tn*10* mutagenesis of SF2
SF171C5	*lacA17 lacR1 hutC1 hutU*::Tn*917-lacZ trpC2 adh1*::Tn*10*	SF170C5 × SF21 DNA
SF172C5	*lacA17 lacR1 hutC1 hutU*::Tn*917-lacZ trpC2 adh5*::Tn*10*	SF170C5 × SF25 DNA
SF173C5	*lacA17 lacR1 hutC1 hutU*::Tn*917-lacZ trpC2 adh7*::Tn*10*	SF170C5 × SF27 DNA
SF174C5	*lacA17 lacR1 hutC1 hutU*::Tn*917-lacZ trpC2 adh10*::Tn*10*	SF170C5 × SF210 DNA
SF175C5	*lacA17 lacR1 hutC1 hutU*::Tn*917-lacZ trpC2 adh14*::Tn*10*	SF170C5 × SF214 DNA
SF176C5	*lacA17 lacR1 hutC1 hutU*::Tn*917-lacZ trpC2 adh17*::Tn*10*	SF170C5 × SF217 DNA
SF170C5Y	*lacZ17 lacR1 hutC1 hutU*::Tn*917-lacZ trpC2* Δ*amyE*::(*spc codY*)	SF170C5 × PS58 DNA
SF171C5Y	*lacA17 lacR1 hutC1 hutU*::Tn*917-lacZ trpC2 adh1*::Tn*10* Δ*amyE*::(*spc codY*)	SF171C5 × PS58 DNA
SF172C5Y	*lacA17 lacR1 hutC1 hutU*::Tn*917-lacZ trpC2 adh5*::Tn*10* Δ*amyE*::(*spc codY*)	SF172C5 × PS58 DNA
SF173C5Y	*lacA17 lacR1 hutC1 hutU*::Tn*917-lacZ trpC2 adh7*::Tn*10* Δ*amyE*::(*spc codY*)	SF173C5 × PS58 DNA
SF174C5Y	*lacA17 lacR1 hutC1 hutU*::Tn*917-lacZ trpC2 adh10*::Tn*10* Δ*amyE*::(*spc codY*)	SF174C5 × PS58 DNA
SF175C5Y	*lacA17 lacR1 hutC1 hutU*::Tn*917-lacZ trpC2 adh14*::Tn*10* Δ*amyE*::(*spc codY*)	SF175C5 × PS58 DNA
SF176C5Y	*lacA17 lacR1 hutC1 hutU*::Tn*917-lacZ trpC2 adh17*::Tn*10* Δ*amyE*::(*spc codY*)	SF176C5 × PS58 DNA

[a] The genotype symbols are those of Anagnostopoulos et al. (1), with the addition of *adh* for amino acid-derepressed *hut* expression.
[b] The (*hut-lacZ*)*626* fusion contains the $hutO_{CR2}$ DNA fragment from positions +76 to +261 cloned between the *gcaD* promoter and the *lacZ* gene in pSFL2 (28).

lactosidase activity present in *B. subtilis* 168 cells containing the promoterless *lacZ* gene from either pSFL1 or pSFL2 integrated at the *amyE* site. Proline oxidase, α-glucosidase, gluconate kinase, and inositol dehydrogenase were assayed as described previously (9, 10). Protein concentrations were determined by the method of Lowry et al. (15), with bovine serum albumin as a standard.

Isolation of *trans*-acting *adh* mutants deficient in *hut* amino acid repression. Eight independent libraries of mini-Tn*10* insertions in strain SF2 (*gltA*) were isolated, as previously described (20), and plated onto Luria-Bertani plates (26) containing 5 μg of chloramphenicol per ml so that each plate contained 30 to 400 individual colonies. Luria-Bertani–chloramphenicol plates were incubated at 50°C overnight and replica plated onto citrate minimal BSS-chloramphenicol plates (2) containing 0.2% histidine and the 13-amino-acid mixture as nitrogen sources. A total of 3,000 to 5,000 colonies were replica plated for each transposon library. Mutant colonies which used histidine as a glutamate source during growth in the presence of amino acids appeared after 24 h of incubation at 37°C and were purified by two rounds of single-colony isolation. Transposons present in chromosomal DNAs isolated from these mutant strains were transformed into strain SF170C5 (*hutC1 hutU*::Tn*917-lacZ*) by selecting for the chloramphenicol resistance (Cm^r) gene present on the mini-Tn*10* transposon. Growth of the resulting Cm^r transformants was examined on citrate minimal BSS plates containing 20 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml, glutamine, and the 16-amino-acid mixture. After 2 days of incubation at 37°C, strain SF170C5 formed white colonies on these plates, while mutants deficient in amino acid regulation of *hut* expression grew as blue colonies.

RESULTS

Isolation of *trans*-acting mutations which relieve amino acid repression of *hut* expression. To search for *trans*-acting factors

required for amino acid repression of *hut* expression, a method that directly selects for mutants which synthesize high levels of the *hut* enzymes during growth in the presence of amino acids was developed. Because glutamate synthase (*gltAB*) is the only glutamate biosynthetic enzyme in *B. subtilis*, glutamate synthase mutants require glutamate under all growth conditions. Histidine, which is degraded to glutamate and ammonium, can provide sufficient glutamate to satisfy the glutamate growth requirement of *gltA* mutants only when *hut* expression is completely derepressed (8). Thus, mutations which allow *gltA* mutants to utilize histidine as a glutamate source in the presence of amino acids should lie in genes required for amino acid repression of *hut* expression. These mutations have been designated *adh* for amino acid-derepressed *hut* expression.

A mixture of 13 amino acids which represses *hut* expression but does not contain any amino acid that is directly metabolized to glutamate was identified. Strain SF2 (*gltA*) is unable to grow on citrate minimal BSS plates containing 0.2% L-histidine and the 13-amino-acid mixture, while Glt$^+$ strains readily form colonies on this medium. Citrate, which does not exert catabolite repression of *hut* expression, was used as the carbon source in the growth medium to avoid isolating mutants deficient in carbon catabolite repression of *hut* expression.

After mini-Tn*10* mutagenesis of strain SF2, 29 mutant colonies whose glutamate growth requirement could be satisfied by histidine during growth in the presence of the 13-amino-acid mixture were isolated. To identify transposon insertions that were genetically linked to the Adh phenotype, DNAs from 18 of these *adh* mutants were used to transform strain SF170C5 (*hutC1 hutU*::Tn*917-lacZ*) to chloramphenicol resistance and the colony color of resulting transformants was examined on citrate minimal BSS–X-Gal plates containing the 16-amino-acid mixture. SF170C5 grows as white colonies on these plates. Ten transposon insertions that caused SF170C5 to form blue colonies were identified. The *hutC1* mutation in strain SF170C5 causes the *hut* operon to be expressed in the absence of histidine. Since histidine transport is not required for *hut* expression in strain SF170C5, amino acid repression occurs only at the $hutO_A$ site (27). Thus, the *adh* mutations identified by using strain SF170C5 (*hutC1*) relieve amino acid repression mediated at the $hutO_A$ operator.

Six of the *adh* strains, SF171C5, SF172C5, SF173C5, SF174C5, SF175C5, and SF176C5, each of which was derived from an independent transposon library, were further characterized. An examination of β-galactosidase expression from the *hutU*::Tn*917-lacZ* insertion in strain SF170C5 and the isogenic *adh* mutants showed that the amino acid repression of *hut* expression seen in strain SF170C5 was significantly relieved in *adh* mutants (Table 2 and data not shown).

Genetic characterization of *adh* mutants. All 10 of the *adh* transformants of SF170C5 grew as small pale colonies on Difco sporulation medium plates. Since mutations in the *codVWXY* operon that relieve nutritional regulation of *dpp* expression have the same growth phenotype on this medium (25), the *adh* insertions may have occurred in the *codVWXY* operon. This hypothesis was confirmed by showing that the *adh* transposons are genetically linked to *codX*. Chromosomal DNAs from the six *adh* mutant strains, SF171C5, SF172C5, SF173C5, SF174C5, SF175C5, and SF176C5, were used to transform strain SF20 (*codX*::*neo*) to Cm^r with limiting amounts of *adh* chromosomal DNA, and the resulting Cm^r transformants were screened for Neo^r. Since 94 to 100% of the Cm^r transformants of SF20 (*codX*::*neo*) obtained with each of the six *adh* chromosomal DNAs were also Neo^s, all six *adh* transposon insertions are tightly linked to *codX*.

Only the *codY* gene in the *codVWXY* operon is required for nutritional regulation of the *dpp* operon (25). To determine whether a defect in *codY* expression is responsible for the altered amino acid repression of *hut* expression seen in *adh* mutant strains, the ability of *codY* to complement the amino acid phenotype of *adh* mutants in *trans* was examined. The six *adh* mutants were transformed to spectinomycin (*spc*) resistance with chromosomal DNA from strain PS58, which contains copies of the *codY* and *spc* genes integrated at the *amyE* locus (21). The resulting transformants, SF171C5Y, SF172C5Y, SF173C5Y, SF174C5Y, SF175C5Y, and SF176C5Y, all grew as white colonies on citrate minimal X-Gal plates containing the 16-amino-acid mixture, while their parental strains formed blue colonies on this medium. An examination of β-galactosidase levels in these strains showed that the wild-type copy of the *codY* gene at the *amyE* locus restored amino acid repression of *hut* expression in all of the *adh* mutant strains (Table 2 and data not shown). Interestingly, the level of amino acid repression of *hut* expression was almost threefold higher in strain SF170C5Y, which contains two copies of *codY*, than in SF170C5, which has only one *codY* gene (Table 2).

TABLE 2. β-Galactosidase levels in wild-type and *adh*::Tn*10* strains

Strain[a]	Relevant genotype	β-Galactosidase sp act (U/mg of protein) on[b]:		Amino acid repression ratio[c]
		Citrate	Citrate + AA	
SF170C5	Wild type	62.2	6.6	9.4
SF170C5Y	*amyE*::(*spc codY*)	62.2	2.4	25.9
SF171C5	*adh1*::Tn*10*	61.1	44.4	1.4
SF171C5Y	*adh1*::Tn*10 amyE*::(*spc codY*)	65.7	5.2	12.6
SF172C5	*adh5*::Tn*10*	64.4	32.2	2.0
SF172C5Y	*adh5*::Tn*10 amyE*::(*spc codY*)	58.2	5.8	10.0
SF173C5	*adh7*::Tn*10*	74.9	35.0	2.1
SF173C5Y	*adh7*::Tn*10 amyE*::(*spc codY*)	68.4	2.6	26.3
SF174C5	*adh10*::Tn*10*	78.9	35.0	2.2
SF174C5Y	*adh10*::Tn*10 amyE*::(*spc codY*)	69.6	5.0	13.9

[a] All strains are SF170C5 derivatives containing the *hutU*::Tn*917-lacZ* insertion and the *hutC1* mutation.

[b] Data are averages of two to five determinations which did not vary by more than 20%. Cultures were grown in MOPS minimal medium (17) containing citrate and glutamine as the carbon and nitrogen sources, respectively, with the indicated additions. AA, the 16-amino-acid mixture (2).

[c] The amino acid repression ratio was calculated by dividing the enzyme activity found in cultures grown without amino acids by the enzyme activity found in amino acid-grown cultures.

Amino acid repression of histidine and proline utilization in Δ*codY* mutants. Amino acid regulation of *hut* expression occurs by two different mechanisms, inhibition of the transport of the *hut* inducer, histidine, and repression of *hut* transcription at the $hutO_A$ operator (27). To determine whether CodY participates in both mechanisms of *hut* amino acid repression, the effects of the Δ*codY* mutation on the synthesis of histidase, the first enzyme in the histidine-degradative pathway, were examined in wild-type and *hutC1* genetic backgrounds.

In citrate-grown cultures, the level of amino acid repression of histidase expression seen in SF168Y (Δ*codY*) cultures was 10-fold lower than that seen in 168 (wild-type) cultures (Table 3). Almost no amino acid repression occurs in strain SF168CY (Δ*codY hutC1*), in which the *hutC1* mutation allows the *hut* operon to be expressed in the absence of the inducer, histidine (Table 3). These results suggest that CodY is required only for amino acid repression mediated at the $hutO_A$ operator.

This was confirmed by using *hut-lacZ* fusions that allow amino acid repression mediated at the $hutO_A$ operator and amino acid inhibition of the histidine transport to be examined

TABLE 3. Histidase levels in wild-type, *hutC1*, and Δ*codY* strains

Strain[a]	Relevant genotype	Histidase sp act (U/mg of protein) on[b]:			Catabolite repression ratio[c]	Amino acid repression ratio[d]
		Glucose	Citrate	Citrate + AA		
168	Wild type	4.5	148	0.4	32.9	370
SF168Y	Δ*codY*	22.1	190	5.4	8.6	35.2
SF168C	*hutC1*	6.1	100	9.9	16.4	10.1
SF168CY	*hutC1* Δ*codY*	19.6	122	61.7	6.2	2.0

[a] All strains are 168 derivatives.
[b] Data are averages of two to five determinations which did not vary by more than 20%. Cultures were grown in MOPS minimal medium containing glutamine as the nitrogen source with the indicated additions. Expression of the *hut* operon was induced by adding 0.1% histidine to the growth medium for strains 168 and SF168Y, which do not contain the *hutC1* mutation. AA, the 16-amino-acid mixture (2).
[c] The catabolite repression ratio was calculated by dividing the enzyme activity found in citrate-grown cultures by the enzyme activity found in glucose-grown cultures.
[d] See Table 2, footnote *c*.

separately. Amino acid regulation occurs only at the $hutO_A$ operator in the (*hut-lacZ*)*603* fusion, which contains the *hut* promoter and $hutO_A$ operator (Fig. 1). Amino acid repression of the (*hut-lacZ*)*603* fusion is almost completely relieved in the Δ*codY* mutant (Table 4). In the (*hut-lacZ*)*624* fusion, the *hut* terminator was cloned between the heterologous *gcaD* promoter (27) and *lacZ* gene (Fig. 1). Histidine-dependent induction of *hut* expression results from antitermination at the *hut* terminator. Thus, the (*hut-lacZ*)*624* fusion can be used to study *hut* induction and to indirectly examine the inhibition of histidine transport in amino acid-grown cultures. Similar levels of β-galactosidase expression from the (*hut-lacZ*)*624* fusion were seen in wild-type and Δ*codY* cultures (Table 4). These results indicate that CodY is required only for amino acid repression mediated at the $hutO_A$ site.

To determine whether CodY participates in the amino acid repression of proline utilization, the levels of proline oxidase, the first enzyme in proline degradation, were determined in extracts of 168 (wild-type) and SF168Y (Δ*codY*) cultures grown in glucose minimal medium with and without the 16-amino-acid mixture. Proline (0.1%) was added to these cultures to induce the expression of the proline-degradative enzymes. No defect in the amino acid regulation of proline oxidase expression was observed in Δ*codY* cultures (data not shown).

Carbon catabolite repression in *codY* mutants. Both amino acid repression and glucose repression of the *dpp* operon are relieved in *codY* mutants (23) (Table 4). An examination of histidase expression in wild-type and Δ*codY* mutants indicated that glucose repression of *hut* expression was partially deficient in Δ*codY* mutants (Table 3). Catabolite repression of *hut* expression occurs at two sites in the *hut* operon, $hutO_{CR1}$ and $hutO_{CR2}$ (28). To determine whether CodY participates in catabolite repression mediated at both $hutO_{CR}$ sites, β-galactosidase expression from various *hut-lacZ* fusions was examined in wild-type and Δ*codY* mutant strains. The (*hut-lacZ*)*603* fusion contains the *hut* promoter and only the $hutO_{CR1}$ site, while the *hut* DNA in the (*hut-lacZ*)*607* fusion contains both $hutO_{CR}$ sites (Fig. 1). The *codY* mutation completely relieved catabolite repression of β-galactosidase expression from the (*hut-lacZ*)*603* fusion but only partially relieved glucose repression of the (*hut-lacZ*)*607* fusion (Table 4). The (*hut-lacZ*)*626* fusion contains the $hutO_{CR2}$ operator cloned between the heterologous *gcaD* promoter and *lacZ* gene (Fig. 1) (28). No defect in catabolite repression of β-galactosidase expression from the (*hut-lacZ*)*626* fusion was seen in the Δ*codY* mutant (Table 4). These results indicate that CodY participates only in catabolite repression mediated at the $hutO_{CR1}$ site.

To determine whether a general defect in catabolite repression is present in *codY* mutants, the expression of enzymes involved in gluconate, inositol, and maltose degradation was examined in 168 (wild-type) and SF168Y (Δ*codY*) strains. Similar levels of glucose repression for gluconate kinase, inositol dehydrogenase, and α-glucosidase expression were observed in strains 168 (wild type) and SF168Y (Δ*codY*) (data not shown).

Expression of the *hut* and *dpp* promoters in cultures grown in various media. The levels of β-galactosidase expression were determined in cultures of SF168CD (*dpp-lacZ*) and SF603C [(*hut-lacZ*)*603*] grown with different carbon and nitrogen sources (Table 5). These data reveal two novel observations regarding the regulation of these gene fusions. First of all, the expression of both fusions is regulated in response to nitrogen availability. The level of *dpp* expression is 3.5-fold higher in cells grown with a limiting nitrogen source, γ-aminobutyric acid, than in cells grown with a good nitrogen source, glutamine. The (*hut-lacZ*)*603* fusion was regulated 1.8-fold under the same growth conditions. To determine whether CodY or another regulatory system is responsible for the elevated expression of these fusions in nitrogen-limited cells, the effects of nitrogen limitation on β-galactosidase expression from the *dpp-lacZ* and (*hut-lacZ*)*603* fusions were examined in Δ*codY* cultures. Similar levels of β-galactosidase expression from the *dpp-lacZ* and (*hut-lacZ*)*603* fusions were seen in Δ*codY* cultures grown in glucose minimal medium containing either glutamine or γ-aminobutyric acid as the sole nitrogen source (data not shown). This indicates that the elevation of β-galactosidase expression from the *dpp-lacZ* and (*hut-lacZ*)*603* fusions seen in wild-type nitrogen-limited cultures most likely results from CodY-dependent regulation. Thus, CodY regulation responds not only to carbon and amino acid availability but also to nitrogen availability.

Secondly, the data in Table 5 indicate that the levels of expression of the *dpp-lacZ* and (*hut-lacZ*)*603* gene fusions are inversely correlated with the growth rates of cultures. Cultures with the fastest doubling times had the lowest levels of β-galactosidase, while higher levels of β-galactosidase were present in cultures grown in media that resulted in slower doubling times. Since the same pattern of regulation was observed for

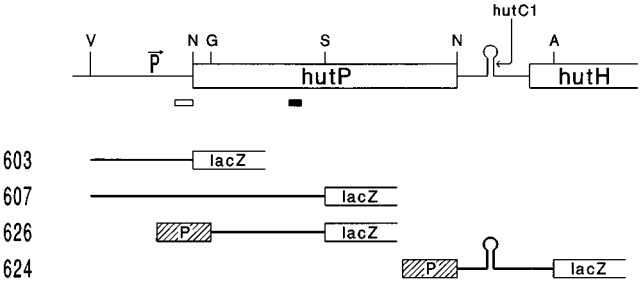

FIG. 1. Physical structure of the *hut* operon. The *hut* DNA fragments used in the construction of *hut-lacZ* fusions are indicated below the operon map. The open rectangle below the operon map indicates the location of the $hutO_{CR1}$ and $hutO_A$ sites. The $hutO_{CR2}$ site is indicated by a solid rectangle. The *hut* promoter is indicated by the letter P with an arrow above it. The *gcaD* promoter present in several *hut-lacZ* fusions is indicated by a crosshatched rectangle. Restriction site abbreviations: A, *Aat*II; G, *Nae*I; N, *Nde*I; V, *Eco*RV; and S, *Nsp*I. Other abbreviations: HutP, *hut*-regulatory protein; *hutH*, histidase structural gene; *hutC1*, mutation in the *hut* terminator that results in constitutive *hut* expression; and *lacZ*, β-galactosidase gene.

TABLE 4. β-Galactosidase levels in wild-type and Δ*codY* strains containing various *lacZ* fusions

Strain[a]	Relevant genotype	*lacZ* fusion[b]	β-Galactosidase sp act (U/mg of protein) on[c]:			Catabolite repression ratio[d]	Amino acid repression ratio[e]
			Glucose	Citrate	Citrate + AA		
SF168CD	Wild type	*dpp*	7.7	25.1	1.5	3.3	16.7
SF168CDY	Δ*codY*	*dpp*	34.2	44.4	34.2	1.3	1.3
SF603C	Wild type	*hut603*	12.8	26.9	2.3	2.1	11.7
SF603CY	Δ*codY*	*hut603*	27.5	33.1	26.0	1.2	1.3
SF607C	Wild type	*hut607*	1.3	25.7	ND[f]	19.7	
SF607CY	Δ*codY*	*hut607*	4.7	32.8	ND	7.0	
SF620C	Wild type	*hut620*	34.2	32.6	46.6	1.0	0.7
SF620CY	Δ*codY*	*hut620*	30.9	35.8	49.2	1.2	0.7
SF626C	Wild type	*hut626*	0.6	11.8	ND	19.6	
SF626CY	Δ*codY*	*hut626*	0.5	11.1	ND	22.2	
SF624C	Wild type	*hut624*	ND	5.3	1.0		7.6
SF624CY	Δ*codY*	*hut624*	ND	5.3	0.9		8.4

[a] All strains are 168 derivatives.
[b] All *lacZ* fusions were integrated as a single copy at the *amyE* locus.
[c] Data are averages of two to five determinations which did not vary by more than 20%. Cultures were grown in MOPS minimal medium containing glutamine as the nitrogen source with the indicated additions. Histidine (0.1%) was added to the growth medium of strains containing *hut-lacZ* fusions to induce their expression.
[d] The catabolite repression ratio was calculated as described in Table 3, footnote *c*, except that the ratio was corrected for repression observed with the (*hut-lacZ*)*620* fusion, which contains the *gcaD* promoter cloned directly upstream of the *lacZ* gene.
[e] The amino acid repression ratio was calculated as described in Table 2, footnote *c*, except that the ratio was corrected for repression observed with the (*hut-lacZ*)*620* fusion.
[f] ND, not determined.

both *dpp-lacZ* and (*hut-lacZ*)*603* gene fusions, CodY appears to regulate gene expression in response to growth rate during exponential growth.

DISCUSSION

Our previous analysis of the *hut* operon indicated that amino acid regulation of *hut* expression results from inhibition of the uptake of L-histidine, the *hut* inducer, and repression of *hut* transcription at the $hutO_A$ operator (27). Since CodY, which represses transcription of the *dpp* operon, also negatively regulates *hut* expression at the $hutO_A$ site, the regulation of these two operons partially overlaps. An examination of the nucleotide sequences of the $hutO_A$ and *dpp* promoter regions did not reveal any common inverted repeat sequence that might function as an operator. This suggests that CodY binds to a nonpalindromic sequence motif. Indeed, Serror and Sonenshein (22) have proposed that CodY recognizes and binds to a three-dimensional structure formed by AT-rich DNA. It is also possible that CodY only indirectly mediates regulation at the $hutO_A$ site and that the true $hutO_A$-binding protein has not yet been identified.

The observation that no defect in the amino acid regulation of proline utilization or the histidine-dependent induction of *hut* expression could be detected in *codY* mutants indicates that additional mechanisms of amino acid repression are present in *B. subtilis*. An examination of the amino acid repression of the transport of L-histidine, the *hut* inducer, showed that this regulation is complex (27). Synthesis of the *B. subtilis* histidine permease(s) is regulated in response to amino acids. In addition, histidine uptake in amino acid-grown cells is posttranslationally inhibited by amino acids. At this time, it is unclear to what extent each of these phenomena contributes to amino acid repression of histidine transport. To determine whether the regulation of expression of histidine transport systems is required for amino acid repression of histidine transport, it will be necessary to identify and examine the expression of genes encoding histidine uptake and export systems in *B. subtilis*. It may be relevant that the final gene in the *hut* operon, *hutM*, has been proposed to encode a histidine permease (29). If *hutM* transcription occurred only from the *hut* promoter, then its expression would be subject to regulation by amino acid repression.

Proline utilization is regulated only by induction and amino acid repression in *B. subtilis* (2). Since amino acid repression of the transport of histidine, the *hut* inducer, prevents induction of the *hut* operon in amino acid-grown cells, it is tempting to speculate that inducer exclusion is also involved in amino acid repression of proline degradation. Proline uptake has been previously examined in *B. subtilis* membrane vesicles (14). These studies showed that proline uptake was inhibited almost 2-fold when a 200-fold excess of either glycine, asparagine, glutamine, aspartate, or glutamate was present in the uptake mixture. Since these five amino acids are present in the 16-amino-acid mixture used to exert amino acid repression, competitive inhibition of proline uptake may be responsible, in part, for amino acid repression of proline utilization.

The results of our previously published deletion analysis of the *hut* operon suggested that the following two distinct operators lie immediately downstream of the *hut* promoter: (i) the $hutO_{CR1}$ site, which mediates low-level carbon catabolite re-

TABLE 5. β-Galactosidase levels in wild-type cells containing the *dpp-lacZ* and (*hut-lacZ*)*603* fusions

Growth medium[a]		Doubling time (min)	β-Galactosidase sp act (U/mg of protein) in[b]:	
Carbon source	Nitrogen source		SF168CD (*dpp-lacZ*)	SF603C [(*hut-lacZ*)*603*]
Glucose	Glutamine + AA	37	0.2	0.5
Citrate	Glutamine + AA	48	1.8	2.0
Glucose	Glutamine	56	6.7	10.9
Glucose	GABA	123	23.8	19.9
Citrate	Glutamine	140	25.3	25.3

[a] Cultures were grown in MOPS minimal medium containing 0.1% histidine (to induce the *hut* operon) and the indicated carbon and nitrogen sources. Abbreviations: GABA, γ-aminobutryic acid; AA, the 16-amino-acid mixture (2).
[b] Data are averages of two to six determinations which did not vary by more than 20%.

pression; and (ii) the $hutO_A$ site, which is partially responsible for *hut* amino acid repression (27, 28). The observation that both carbon catabolite repression and amino acid repression of the (*hut-lacZ*)*603* fusion were relieved in the Δ*codY* mutant (Table 4) argues that CodY mediates both carbon catabolite repression and amino acid repression at a single operator. To resolve these seemingly contradictory observations, we are currently performing a mutational analysis of the $hutO_A$ and $hutO_{CR1}$ operators.

The observation that *codY* mutations relieve catabolite repression mediated in the *hut* promoter region but not catabolite repression at the $hutO_{CR2}$ site indicates that two different systems regulate *hut* expression in response to carbon availability. There is genetic evidence for multiple systems of glucose catabolite repression in *B. subtilis*. For example, the *ccpA* and *ptsH1* mutations relieve carbon catabolite repression of the expression of gluconate kinase and glucitol dehydrogenase, but not of α-glucosidase and glycerol kinase (6). The nucleotide sequences of $hutO_{CR2}$ and of sites required for catabolite repression of the *amyE*, *gnt*, and *xyl* genes have strong sequence similarities to each other, but not with the catabolite repression site for aconitase (11, 28).

If carbon, nitrogen, and amino acid repression of *dpp* expression is mediated by CodY, then the DNA binding activity of the CodY protein could be regulated in response to multiple signals. Each signal would reflect the availability of a particular class of nutrients. Alternatively, CodY activity may be modulated by a single signal that does not directly respond to carbon, nitrogen, or amino acid availability but instead is produced in response to a parameter such as growth rate. By this scenario, the low-level CodY regulation seen in response to carbon and nitrogen availability would result from the intermediate levels of the CodY-regulatory signal produced under these growth conditions.

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