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# Catabolite Repression of the *Bacillus subtilis hut* Operon Requires a *cis*-Acting Site Located Downstream of the Transcription Initiation Site

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**Expression of the *Bacillus subtilis hut* operon is subject to regulation by catabolite repression. A set of *hut-lacZ* transcriptional fusions was constructed and used to identify two *cis*-acting sites involved in catabolite repression. The $hutO_{CR1}$ operator site lies immediately downstream of the *hut* promoter and weakly regulates *hut* expression in response to catabolite repression. The downstream $hutO_{CR2}$ operator site lies within the *hutP* gene, between positions +203 and +216, and is required for wild-type levels of catabolite repression. Both the $hutO_{CR1}$ and $hutO_{CR2}$ operators have sequence similarity to the sites which mediate catabolite repression of several other *B. subtilis* genes. Two mutations which relieve catabolite repression of *hut* expression were found to alter the nucleotide sequence of the $hutO_{CR2}$ operator. Catabolite repression of *hut* expression was partially relieved in strains containing the *ccpA* mutation but not in strains containing either the *pai* or *hpr* mutation.**

When bacterial cells grow on media containing rapidly metabolized carbohydrates, the synthesis of enzymes and permeases involved in the utilization of other carbon-containing compounds is often inhibited. This regulation of gene expression in response to carbon availability is known as catabolite repression (CR) (35). In enteric bacteria, genes whose transcription is subject to CR are positively regulated by cyclic AMP and the catabolite gene activator protein (37). When complexed with cyclic AMP, the catabolite gene activator protein binds to a specific DNA sequence located upstream of CR-regulated promoters and activates transcription initiation (50).

Although the regulatory system mediating CR in *Bacillus subtilis* is unknown (6, 15), cyclic AMP is unlikely to be the signal mediating this regulation (34, 45, 55). The locations and sequences of the *cis*-acting sites mediating CR of the *B. subtilis amyE* (44, 60), *citB* (17), *dciA* (57), *gnt* (40, 41), and *xyl* (29) genes have been determined. For all five of these genes, mutations which inactivate the *cis*-acting site result in derepressed gene expression during growth in the presence of glucose. This indicates that CR of these *B. subtilis* genes is mediated by a negative regulatory system. The *cis*-acting CR operator sites for the *gnt* (40, 41) and *xyl* (29) operons were found to be located within structural genes downstream of the transcription initiation site.

*B. subtilis* strains with mutations at diverse genetic loci which are pleiotropically altered in CR have been isolated. Mutations in the structural gene for glutamine synthetase, *glnA*, partially relieve CR of aconitase, citrate synthase, histidase, and α-glucosidase (14). The *crsA* mutation, which lies in the gene encoding the $\sigma^A$ subunit of RNA polymerase, causes sporulation and causes the expression of α-amylase, acetoin dehydrogenase, aconitase, and gluconate kinase, but not histidase, to be insensitive to glucose repression (4, 6, 19, 58, 59). CR of aconitase, α-amylase, histidase, and α-glucosidase is partially relieved in strains containing the *cdh-3* mutation (13, 15, 31). The *ccpA* mutation relieves glucose repression of α-amylase, gluconate kinase, and levanase expression (19, 22, 39). The *ccpA* gene encodes a protein whose amino acid sequence is highly similar to the LacI family of repressor proteins (22). Although CcpA is likely to be a DNA-binding protein, it is not known whether CcpA is directly involved in the mechanism of CR (6, 19).

The four enzymes required for histidine utilization (*hut*) in *B. subtilis* are encoded within a single multicistronic operon (7, 30, 36, 48). The first open reading frame in the *hut* operon, *hutP*, encodes a positive regulatory protein required for *hut* operon expression, while the second open reading frame, *hutH*, encodes histidase, the first enzyme in the *hut* degradative pathway (7, 48). Located between the *hutP* and *hutH* genes is a nucleotide sequence which could form a stem-loop structure (see Fig. 1). It has been proposed that the histidine-dependent induction of *hut* operon expression results from transcription antitermination mediated by the HutP protein at this stem-loop structure (48). The *hutP1* missense mutation results in inability to induce *hut* operon expression (7). The *hutC1* mutation results in constitutive expression of the *hut* operon (7) and lies within the stem-loop structure located between the *hutP* and *hutH* genes (63).

Expression of the *B. subtilis hut* operon is severely repressed during growth in media containing glucose, glycerol, or malate as a carbon source (7). Since *hut* expression is still regulated by CR in strains which contain the *hutC1* mutation, histidine transport does not play a major role in CR of *hut* expression (7). Several mutations which relieve CR of *hut* expression and are genetically linked to the *hutH* gene have been isolated (7, 13).

Oda et al. previously reported that CR of *hut* expression is mediated at the *hut* promoter and by transcriptional termination at an inverted repeat located within the *hutP* gene (47). In this study, the CR operator at the *hut* promoter was identified. In addition, a site within the *hutP* gene with strong sequence similarity to the proposed *B. subtilis* CR consensus sequence (60), rather than the complete inverted repeat structure proposed by Oda et al. (47), was shown to be required for CR of *hut* expression.

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

| Strain | Genotype[a] | Reference, source, or derivation |
|---|---|---|
| 168 | *trpC2* | This laboratory |
| 1A271 | *hutP1 str sul* | BGSC[b] |
| 1A680 | *lacA17 lacR1 trpC2* | BGSC[b] |
| IS708 | *Δhpr::cat leuA8 metB5 hisA1* | I. Smith, A. Grossman; 49 |
| IS75*Δpai::cat* | *Δpai::cat leuA8 metB5 hisA1* | I. Smith |
| SF168R | *hutO*$_{CR2}$*4 trpC2* | 3 |
| SF168C | *hutC1 trpC2* | 3 |
| TH256 | *ccpA*::Tn*917-lacZ lys-3 trpC2 metB10* | T. Henkin |
| SF256C | *hutC1 ccpA*::Tn*917-lacZ trpC2* | SF168C × TH256 DNA |
| SF1686 | *hutU*::Tn*917-lacZ*::pTV21Δ2 *trpC2* | 2 |
| SF406 | *hutH*::pHUT406 *erm trpC2* | 168 × pHUT406 |
| SF406R | *hutO*$_{CR2}$*4 hutH*::pHUT406 *erm trpC2* | SF168R × pHUT406 |
| SF406P | *hutP1 hutH*::pHUT406 *erm str sul* | 1A271 × pHUT406 |
| SF1210 | *ΔhutPH::neo trpC2* | 168 × pHUT411 |
| SF708 | *Δhpr::cat trpC2* | 168 × IS708 DNA |
| SF709 | *Δpai::cat trpC2* | 168 × IS75*Δpai::cat* DNA |
| SF168C5 | *hutC1 hutU*::Tn*917-lacZ trpC2* | 3 |
| SF170C5 | *lacA17 lacR1 hutC1 hutU*::Tn*917-lacZ trpC2* | 1A680 × SF168C5 DNA |
| SF170CR2 | *lacA17 lacR1 hutC1 hutO*$_{CR2}$*2 hutU*::Tn*917-lacZ trpC2* | Mutagenesis of SF170C5 |
| SF168CR2 | *hutC1 hutO*$_{CR2}$*2 trpC2* | SF1210 × SF170CR2 DNA |
| SF406CR2 | *hutC1 hutO*$_{CR2}$*2 hutH*::pHUT406 *erm trpC2* | SF168CR2 × pHUT406 |

[a] Genotype symbols are those of Anagnostopoulos et al. (1), except for *hutO*$_{CR2}$, which replaces *hutR* (7).
[b] BGSC, *Bacillus* Genetic Stock Center.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** The *B. subtilis* strains used in this study are listed in Table 1. *Escherichia coli* HB101, NM522, MC1061, and KE93, a derivative of MM294 which contains the *pcnB80* mutant allele (32), were used as hosts for DNA cloning experiments.

*E. coli* plasmid pJDC9 was designed to allow cloning of DNA fragments with strong promoter activity and contains a polylinker cloning region flanked by transcriptional terminators (8). pJDC9 confers erythromycin resistance on both *E. coli* and *B. subtilis*, although it does not replicate in *B. subtilis*. Two derivatives of pJDC9 were constructed during the course of this work; pLEW417 and pLEW424 are identical to pJDC9, except that they contain the polylinker regions from pIC7 (38) and pMTL20P (5), respectively.

**Cell growth and media.** The methods used for bacterial cultivation have been previously described (2). The morpholinepropanesulfonic acid (MOPS) minimal medium of Neidhardt et al. (43) was 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 was freshly prepared, filter sterilized, and added at 0.1% to induce the histidine-degradative enzymes. The growth phenotype of the *ccpA* mutant was examined on BSS minimal plates (7) made with Noble agar (Difco Laboratories, Detroit, Mich.). Production of α-amylase was determined on starch plates as previously described (22).

**Enzyme assays.** Extracts for enzyme assays were prepared from cells grown to the mid-log growth phase (70 to 90 Klett units) as previously described (2). Histidase and β-galactosidase were assayed in crude cell extracts as described previously (2). One unit of histidase activity produced 1 nmol of urocanic acid per min. One unit of β-galactosidase activity produced 1 nmol of *o*-nitrophenol per min. β-Galactosidase activity was always corrected for the endogenous β-galactosidase activity present in 168 cells containing the promoterless *lacZ* gene from either pSFL1 or pSFL2 integrated at the *amyE* site. Protein concentration was determined by the method of Lowry et al. (33), with bovine serum albumin as the standard.

**Cloning of *hut* operon DNA.** The chromosomal *hut* DNA upstream of the previously described *hutU*::Tn*917-lacZ* insertion (2) was cloned by using integration plasmid pTV21Δ2 (65). Plasmid pHUT3 was obtained by digesting chromosomal DNA from strain SF1686 with *Hin*dIII. pHUT3 contains 6 kb of DNA upstream of the Tn*917-lacZ* insertion (see Fig. 1). Transformants of *E. coli* HB101 and NM522 which contain pHUT3 grow very slowly, and only very low yields of the plasmid DNA could be obtained. Subsequent experience has revealed that *E. coli* plasmids which contained a complete or amino-terminal portion of the *hutP* gene are unstable and can give rise to promoter mutations and deletions (63). To overcome this problem, subclones of pHUT3 were constructed in pJDC9 or one of its derivatives described above. In addition, these subclones were propagated in *pcnB80* mutant *E. coli* KE93. The *pcnB80* mutation lowers the copy number of ColE1-derived plasmids 16-fold (32) and thus facilitates the cloning of genes which are toxic in *E. coli* (65).

Plasmid pHUT410 (see Fig. 1) was constructed by cloning a 2.5-kb *Sal*I-*Eco*RI DNA fragment containing the *hut* regulatory region from pHUT3 into pJDC9. In plasmid pHUT411 (Fig. 1), the *hut* DNA between the *Eco*RV site upstream of the promoter and the *Bgl*II site in the *hutH* gene in pHUT410 was replaced with the neomycin resistance gene from pBEST501 (27). pHUT406 (see Fig. 1) was constructed by cloning the 530-bp *Bgl*II fragment containing the 5′ end of the *hutH* gene into pLEW417. *B. subtilis* SF406, which contains plasmid pHUT406 integrated at the *hut* chromosomal locus (by single crossover), was constructed by transforming strain 168 to erythromycin resistance with pHUT406. By the method described for cloning chromosomal DNA sequences adjacent to plasmid integrations (65), pHUT450 (see Fig. 1) was isolated from SF406 chromosomal DNA digested with *Eco*RV. pHUT451 and pHUT456 are identical to pHUT450, except that they contain the *hutO*$_{CR2}$*4* and *hutP1* mutations, respectively.

**Construction of *lacZ* transcriptional fusions.** Two *lacZ* transcriptional fusion vectors that integrate at the *amyE* locus were used in these studies. pSFL1 has unique *Eco*RI and

*Hin*dIII restriction sites located upstream of a promoterless *E. coli trpA-lacZ* translational fusion (11, 62). pDH32 is a derivative of ptrpBG1 (56) and has unique *Eco*RI and *Bam*HI restriction sites located upstream of an *spoVG-lacZ* translational fusion (23). The pSFL2 *lacZ* vector was constructed by inserting the following complementary synthetic oligodeoxynucleotides between the *Eco*RI and *Bam*HI sites of pDH32:

5′-AATTCTAGATGCATAAGCTTA-3′
3′-GATCTACGTATTCGAATCTAG-5′

pSFL2 has unique *Eco*RI and *Hin*dIII restriction sites located upstream of a promoterless *spoVG-lacZ* gene fusion.

DNA restriction fragments containing the *hut* promoter were cloned into the polylinker cloning region of pJDC9 or one of its derivatives, pLEW417 or pLEW424. The *Eco*RI-*Hin*dIII *hut* promoter DNA fragments from these clones were inserted into pSFL1 to construct *hut-lacZ* transcriptional fusions (see Fig. 1).

The *Hin*dIII site downstream of the $P_{SPAC}$ promoter (64) in pAG58 (28) was converted into a *Bam*HI site by using oligodeoxynucleotide adapters (New England BioLabs, Inc.). The same procedure was used to convert the *Hin*dIII site downstream of the $P_{gcaD}$ (formerly *tms*) (26, 46) promoter in pFW5A (61) into a *Bam*HI site. *Eco*RI-*Bam*HI DNA fragments of the $P_{SPAC}$ and $P_{gcaD}$ promoters were subcloned into pLEW424. DNA fragments containing the $hutO_{CR2}$ operator were then inserted into the polylinker region downstream of the promoters. To construct *lacZ* fusions, *Eco*RI-*Hin*dIII fragments containing the $P_{SPAC}$-$hutO_{CR2}$ and $P_{gcaD}$-$hutO_{CR2}$ promoters were cloned into pSFL2. All *lacZ* fusions were integrated by double crossover at the *amyE* locus (56) and screened for loss of α-amylase activity on starch plates (22).

**DNA sequencing.** The dideoxynucleotide chain termination method (54) was used to determine the nucleotide sequence of the cloned *hut* DNA in pHUT450, pHUT451, and pHUT456. Sequencing reactions were performed at 70°C by using *Taq* DNA polymerase (TaqTrack; Promega Corp.) with double-stranded plasmid DNA as the template and $^{32}$P-end-labeled oligodeoxynucleotide primers. All three plasmids were sequenced from the *Eco*RV site upstream of the promoter to nucleotide position +616 in the 5′ end of the *hutH* gene. The sequence of the wild-type *hut* operon DNA and the *hutP1* mutation agreed with previously published data (48).

**Isolation of the $hutO_{CR2}2$ mutation.** Strain SF170C5 was mutagenized with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine as previously described (12) and plated onto BSS minimal plates containing glucose, glutamate, and $NH_4^+$ as carbon and nitrogen sources and 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside per ml. After 2 days of incubation at 37°C, strain SF170C5 forms light blue colonies on this medium. Mutant colonies which expressed derepressed levels of β-galactosidase in the presence of glucose were identified by their dark blue color and purified by two rounds of single-colony isolation. In transformation experiments, the mutation responsible for the altered phenotype in strain SF170C2 was found to be 90% linked to the erythromycin resistance gene present in the *hutU*::Tn*917-lacZ* transposon insertion. This mutation, $hutO_{CR2}2$, was transferred into the 168 strain background by transforming strain SF1210 (Δ*hutPH*::*neo*) to Hut$^+$ with DNA from strain SF170C2. DNA containing the $hutO_{CR2}2$ mutation was cloned and sequenced as described above for the other *hut* mutations.

**Primer extension analysis.** RNA was isolated from *B. subtilis* cells grown to the mid-log growth phase (70 to 90 Klett units) by extraction with guanidine thiocyanate and CsCl centrifugation (2). Primer extension experiments were performed as previously described (3). Autoradiographs were scanned with a Molecular Dynamics personal densitometer and ImageQuant software. The relative levels of *hutP* RNA quantitated (see Fig. 3) were dependent upon the background value used in the densitometric scanning.

## RESULTS

**Construction and regulation of *hut-lacZ* transcriptional fusions.** Chromosomal DNA upstream of the *B. subtilis hutU*::Tn*917-lacZ* transposon insertion was cloned into *E. coli* plasmids as described by Youngman (65). Additional clones were obtained by using integrational plasmid pHUT406 for chromosomal walking. The physical map of these DNA clones is shown in Fig. 1.

To identify *cis*-acting sites required for CR of *hut* expression,

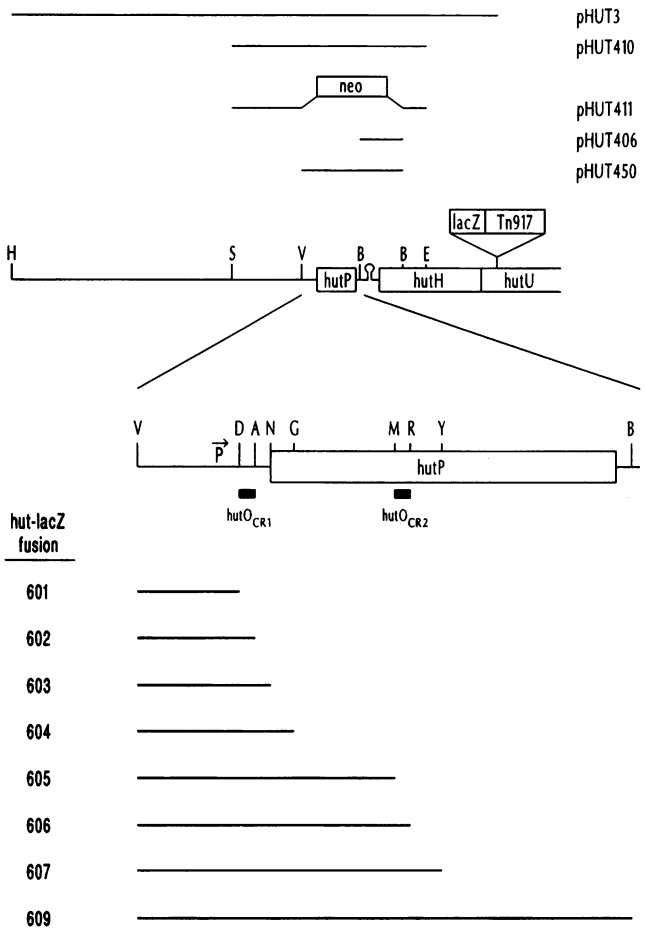


FIG. 1. Physical structure of the *hut* operon. The location of the *hutU*::Tn*917-lacZ* transposon insertion is indicated. The physical maps of the cloned *hut* DNA inserts are shown above the operon map. The DNA fragments used to construct *hut-lacZ* fusions are diagrammed at the bottom. The *hut* promoter is indicated by the letter P with the arrow above it. Restriction enzyme abbreviations: A, *Apo*I; B, *Bgl*II; D, *Dde*I; E, *Eco*RI; G, *Nae*I; H, *Hin*dIII; M, *Mun*I; N, *Nde*I; R, *Bsr*BI; S, *Sal*I; V, *Eco*RV; Y, *Nsp*I. Other abbreviations: *hutP*, *hut* regulatory gene; *hutH*, histidase structural gene; *hutU*, urocanase structural gene; *lacZ*, promoterless β-galactosidase structural gene; *neo*, neomycin resistance gene (27).

TABLE 2. Enzyme levels in *hut-lacZ* fusion strains[a]

| *hut-lacZ* fusion | *hut* DNA endpoint | β-Galactosidase sp act[b] (U/mg of protein) | | Repression ratio[c] | Histidase sp act[b] (U/mg of protein) | | Repression ratio[c] |
|---|---|---|---|---|---|---|---|
| | | Glucose[d] | Citrate[d] | | Glucose[d] | Citrate[d] | |
| pSFL1 | None | 0.044 ± 0.004 | 0.73 ± 0.07 | | 7.4 ± 1.0 | 143 ± 5.0 | 19 |
| 601 | +1 | 3.6 ± 0.1 | 3.4 ± 0.2 | 0.94 | 5.7 ± 0.1 | 180 ± 15 | 32 |
| 602 | +20 | 20.9 ± 2.0 | 39.7 ± 2.0 | 1.9 | 1.4 ± 0.3 | 159 ± 26 | 114 |
| 603 | +42 | 10.9 ± 0.4 | 28.5 ± 2.0 | 2.6 | 1.9 ± 0.2 | 125 ± 7.0 | 66 |
| 604 | +72 | 7.5 ± 1.5 | 26.8 ± 3.5 | 3.6 | 4.1 ± 0.1 | 139 ± 3.0 | 34 |
| 605 | +203 | 11.6 ± 0.1 | 25.3 ± 2.2 | 2.2 | 1.9 ± 0.1 | 111 ± 7.0 | 58 |
| 606 | +223 | 0.71 ± 0.01 | 16.2 ± 0.2 | 23 | 5.7 ± 0.2 | 148 ± 7.0 | 26 |
| 607 | +261 | 0.71 ± 0.06 | 17.7 ± 0.2 | 25 | 5.1 ± 0.3 | 169 ± 10 | 33 |
| 609 | +504 | 1.2 ± 0.1 | 25.8 ± 2.8 | 22 | 18.0 ± 1.0 | 213 ± 15 | 12 |

[a] All strains are 168 derivatives containing the indicated *hut-lacZ* fusion integrated as a single copy at the *amyE* locus.
[b] Average of three to six determinations ± the standard error.
[c] The repression ratio was determined by dividing the enzyme activity present in citrate-grown cultures by the enzyme activity found in glucose-grown cultures.
[d] Cultures were grown in MOPS minimal medium containing either glucose, glutamate, and $NH_4Cl$ or citrate and glutamine as carbon and nitrogen sources. Histidine was added at 0.1% to all of the cultures to induce the *hut* operon.

a set of *hut-lacZ* transcriptional fusions was constructed (Fig. 1). The upstream endpoint of all of the *hut* DNA fragments is the *Eco*RV site located upstream of the *hut* promoter (Fig. 1). The positions of the downstream endpoints are given in Table 2. All of these constructs were integrated into the *B. subtilis* chromosome as single copies at the *amyE* locus. Regulation of β-galactosidase expression from these *hut-lacZ* fusions by CR was examined by growing cells in media containing either glucose or citrate as the carbon source.

The *hut-lacZ* fusions which have downstream endpoints that extend beyond position +223 (606, 607, and 609) were repressed 22- to 25-fold by CR (Table 2). β-Galactosidase expression in cells containing fusions with endpoints between positions +20 and +203 (602, 603, 604, and 605) was repressed 1.9- to 3.6-fold by CR. This low level of CR was not observed in cells containing *hut-lacZ* fusion 601, which has a downstream endpoint at position +1 (Table 2). These results indicate that CR of *hut* expression is mediated by at least two sites.

The $hutO_{CR1}$ operator lies between the endpoints of fusions 601 and 602 (Fig. 2) and is located immediately downstream of the *hut* promoter. As noted previously (60), this sequence has weak similarity to the proposed *B. subtilis* CR operator consensus sequence. The $hutO_{CR1}$ operator contains mismatches with the consensus sequence at three conserved positions (Fig. 2) and is most likely not an intrinsically strong binding site for the *hut* catabolite repressor. The two- to fourfold level of CR observed in cells containing *hut-lacZ* fusions 602, 603, 604, and 605 is most likely due to CR mediated at this site. However, several DNA sequences with weak similarity to the proposed *B. subtilis* CR site lie upstream of the *hut* promoter and are present in these *hut-lacZ* constructs. These upstream sequences may contribute to the low level of CR observed in *hut-lacZ* fusions containing the $hutO_{CR1}$ site.

The $hutO_{CR2}$ operator, which lies between the endpoints of fusions 605 and 606, is highly similar to the CR operator consensus sequence and overlaps the inverted repeat proposed by Oda et al. (47) as the *hut* CR site (Fig. 2). The *hut-lacZ* 606 fusion does not contain most of the downstream portion of this inverted repeat (Fig. 2) but is still regulated by CR to the same extent as the *hut-lacZ* 607 and 609 fusions, which do contain the entire inverted repeat (Table 2). This indicates that the inverted repeat sequence is not required for CR of *hut* expression.

**Promoter-$hutO_{CR2}$-*lacZ* fusions.** To define the $hutO_{CR2}$ site more precisely, we constructed a set of *lacZ* fusions which contain the $hutO_{CR2}$ site cloned between a constitutive *B. subtilis* promoter and the *lacZ* gene. β-Galactosidase expression from the $P_{gcaD}$ (26, 46) and the $P_{SPAC}$ (64) promoters used in these constructions was not significantly regulated by CR (Table 3). However, when a *hut* DNA fragment extending from positions +73 to +261 was cloned downstream of the $P_{gcaD}$ and $P_{SPAC}$ promoters, β-galactosidase expression was repressed 33- and 8.3-fold, respectively, by growth in the presence of glucose (Table 3). The $hutO_{CR2}$ site was more precisely defined by cloning *hut* DNA fragments extending from positions +200 to +261 and +200 to +223 downstream of the $P_{SPAC}$ promoter. Since β-galactosidase expression from both

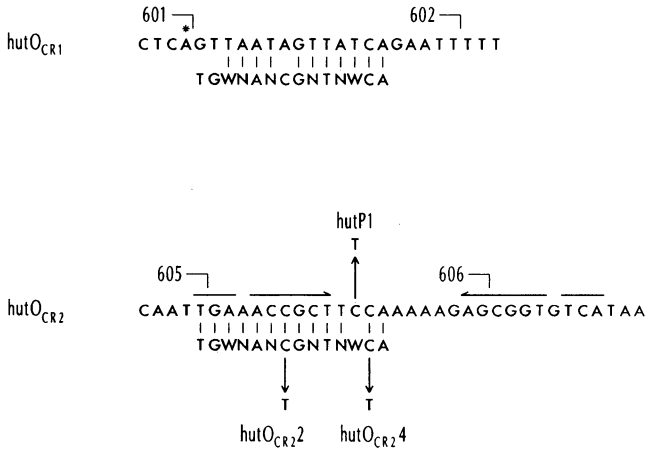


FIG. 2. Sequences of the $hutO_{CR1}$ and $hutO_{CR2}$ sites. The top sequence is the *hut* DNA sequence from positions −3 to +23 along with the downstream endpoints of *hut-lacZ* fusions 601 and 602. The transcription initiation start site is indicated by the asterisk. Aligned below the $hutO_{CR1}$ site is the CR consensus sequence. The bottom sequence is the *hut* DNA sequence from positions +199 to +234 along with the downstream endpoints of *hut-lacZ* fusions 605 and 606. The inverted repeat proposed by Oda et al. (47) as the site of *hut* CR is indicated by the converging arrows above the DNA sequence. The *hutP1* nucleotide lesion is indicated above the DNA sequence. The $hutO_{CR2}2$ and $hutO_{CR2}4$ mutations are indicated below the DNA sequence. Aligned below the $hutO_{CR2}$ site is the CR consensus sequence.

TABLE 3. Enzyme levels in promoter-*hutO*$_{CR2}$-*lacZ* fusion strains[a]

| Promoter | *hutO*$_{CR2}$ DNA fragment endpoints | β-Galactosidase sp act[b] (U/mg of protein) | | Repression ratio[c] | Histidase sp act[b] (U/mg of protein) | | Repression ratio[c] |
|---|---|---|---|---|---|---|---|
| | | Glucose[d] | Citrate[d] | | Glucose[d] | Citrate[d] | |
| $P_{gcaD}$ | None | 17.9 ± 1.0 | 25.2 ± 1.0 | 1.4 | 4.3 ± 0.5 | 122 ± 7 | 28 |
| $P_{gcaD}$ | +73, +261 | 0.26 ± 0.01 | 8.7 ± 0.7 | 33 | 5.5 ± 0.1 | 131 ± 1 | 24 |
| $P_{SPAC}$ | None | 47 ± 2 | 45 ± 9 | 0.95 | 7.4 ± 0.1 | 207 ± 5 | 28 |
| $P_{SPAC}$ | +73, +261 | 1.2 ± 0.1 | 10.0 ± 0.1 | 8.3 | 6.9 ± 0.6 | 177 ± 6 | 26 |
| $P_{SPAC}$ | +200, +261 | 4.5 ± 0.5 | 42 ± 3 | 9.3 | 6.2 ± 0.2 | 183 ± 21 | 30 |
| $P_{SPAC}$ | +200, +223 | 6.0 ± 0.1 | 62 ± 2 | 10.3 | 6.9 ± 0.3 | 205 ± 7 | 30 |

[a] All strains are 168 derivatives containing the indicated promoter-*hutO*$_{CR2}$-*lacZ* fusion integrated as a single copy at the *amyE* locus.
[b] See Table 2, footnote *b*.
[c] See Table 2, footnote *c*.
[d] See Table 2, footnote *d*.

of these $P_{SPAC}$-*hutO*$_{CR2}$-*lacZ* fusions was regulated to same extent by CR (Table 3), the complete *hutO*$_{CR2}$ site must be located between positions +200 and +223.

**Analysis of *hutO*$_{CR2}$ point mutations.** The *hutO*$_{CR2}$*2* and *hutO*$_{CR2}$*4* (formerly *cdh-4*; 13) mutations almost completely relieve CR of histidase expression during growth in the presence of either glucose (Table 4) or malate (data not shown). Nucleotide sequence analysis revealed that both of these mutations lie within the *hutO*$_{CR2}$ operator and change highly conserved bases of the CR consensus sequence (Fig. 2). Although both of these mutations lie within the *hutP* gene, the nucleotide lesions are located in the third position of threonine (*hutO*$_{CR2}$*2*) and serine (*hutO*$_{CR2}$*4*) codons and thus do not alter the amino acid sequence of the HutP protein.

The *hutP1* missense mutation (48) also alters a base pair which lies within the *hutO*$_{CR2}$ operator site (Fig. 2). To analyze the effect of the *hutO*$_{CR2}$*4* and *hutP1* mutations of CR of *hut* expression, *hut-lacZ* 609 fusions containing these mutations were constructed. Initially, these fusions were integrated into the chromosome of strain SF1210 (Δ*hutPH*::*neo*) as single copies at the *amyE* locus. The SF1210 strain was used to prevent homogenotization between the chromosomal *hut* operon and the mutant *hut* sequences in the *hut-lacZ* 609 fusions.

β-Galactosidase expression from the wild-type *hut-lacZ* fusion was repressed 16-fold by growth in the presence of glucose (Table 4). When the *hut-lacZ* fusion contained the *hutO*$_{CR2}$*4* mutation, only 2.8-fold repression of β-galactosidase expression was seen (Table 4). In contrast, the *hutP1-lacZ* 609 fusion was repressed 13-fold by growth in the presence of glucose (Table 4). CR of β-galactosidase expression from the *hutP1-lacZ* fusion was not unexpected. The *hutP1* mutation actually changes the *hutO*$_{CR2}$ site so that it is more similar to the proposed CR consensus sequence (Fig. 2). When the *hut-lacZ* 609 fusions at the *amyE* locus were transferred to a wild-type (*hut*$^+$) genetic background, no significant alteration in their regulation by CR was observed (Table 4).

**Histidase expression in the *hut-lacZ* 609 fusion.** Histidase levels in glucose-grown cells containing both the wild-type and the *hutO*$_{CR2}$*4-lacZ* 609 fusions were higher than those in cultures of strains containing either the *hutP1-lacZ* 609 fusion or the promoterless *lacZ* gene present in pSFL1 (Tables 2 and 4). The *hut-lacZ* 609 fusion is the only fusion that contains the entire coding sequence of the *hutP* gene (Fig. 1). The HutP protein, a positive regulatory factor, has been proposed to mediate histidine-dependent transcriptional antitermination at the stem-loop structure which lies between the *hutP* and *hutH* genes (48). If the levels of the HutP protein are limiting in wild-type strains, then in strains containing the *hut-lacZ* 609 fusion, the second copy of the *hutP* gene would increase HutP levels and thus elevate histidase expression in *trans*. Similarly, because expression of the *hutO*$_{CR2}$*4-lacZ* 609 fusion (and its *hutP* gene) is not significantly repressed by glucose, histidase levels in glucose-grown cultures of strains containing the *hutO*$_{CR2}$*4-lacZ* 609 fusion are higher than in cultures containing the wild-type *hut-lacZ* 609 fusion.

In contrast, β-galactosidase expression from the *hut-lacZ* 609 fusion was not affected by alterations in *hutP* expression or

TABLE 4. Enzyme levels in *hut-lacZ* 609 fusion strains[a]

| *hut-lacZ* 609 fusion genotype | Chromosomal *hut* operon genotype | β-Galactosidase sp act[b] (U/mg of protein) | | Repression ratio[c] | Histidase sp act[b] (U/mg of protein) | | Repression ratio[c] |
|---|---|---|---|---|---|---|---|
| | | Glucose[d] | Citrate[d] | | Glucose[d] | Citrate[d] | |
| | *hutO*$_{CR2}$*2 hutC1* | | | | 145 ± 4 | 175 ± 2 | 1.2 |
| | *hutO*$_{CR2}$*4* | | | | 161 ± 16 | 282 ± 10 | 1.8 |
| *hut*$^+$ | Δ*hutPH*::*neo* | 1.6 ± 0.1 | 25.7 ± 0.6 | 16 | <0.5 | <0.5 | |
| *hutO*$_{CR2}$*4* | Δ*hutPH*::*neo* | 16.7 ± 0.5 | 47.0 ± 2.0 | 2.8 | <0.5 | <0.5 | |
| *hutP1* | Δ*hutPH*::*neo* | 1.0 ± 0.06 | 13.3 ± 1.0 | 13.3 | <0.5 | <0.5 | |
| *hut*$^+$ | *hut*$^+$ | 1.2 ± 0.1 | 25.8 ± 2.8 | 22 | 18.0 ± 1.0 | 213 ± 15 | 12 |
| *hutO*$_{CR2}$*4* | *hut*$^+$ | 17.9 ± 1.0 | 43.5 ± 3.0 | 2.4 | 34.2 ± 3.0 | 232 ± 17 | 6.8 |
| *hutP1* | *hut*$^+$ | 0.8 ± 0.1 | 13.1 ± 0.1 | 16 | 4.3 ± 0.1 | 119 ± 9 | 28 |

[a] All strains are 168 derivatives containing the indicated *hut* chromosomal mutations and *hut-lacZ* 609 fusions integrated as a single copy at the *amyE* locus, except for strains SF168CR2 (*hutO*$_{CR2}$*2 hutC1*) and SF168R (*hutO*$_{CR2}$*4*), which do not contain a *hut-lacZ* fusion.
[b] See Table 2, footnote *b*.
[c] See Table 2, footnote *c*.
[d] See Table 2, footnote *d*.

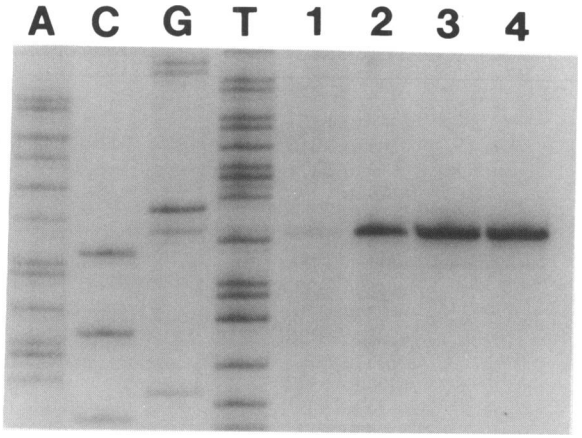


FIG. 3. Primer extension analysis of *hutP* mRNA. A $^{32}$P-end-labeled oligodeoxynucleotide primer complementary to nucleotides +101 to +120 of the *hut* mRNA was used for primer extension analysis (lanes 1 to 4) and for dideoxy sequencing of pHUT410 (lanes A, C, G, and T). RNA was isolated from *B. subtilis* 168 (wild type; lanes 1 and 2) or SF168R (*hutO*$_{CR2}$*4*; lanes 3 and 4) grown in minimal media containing either glucose (lanes 1 and 3) or citrate (lanes 2 and 4) as the carbon source. The histidase specific activities (in units per milligram of protein) measured in extracts of these cultures were 9.6 (lane 1), 174 (lane 2), 134 (lane 3), and 232 (lane 4).

copy number (Tables 2 and 4). Since the *hut-lacZ* 609 fusion does not contain the stem-loop structure located between the *hutP* and *hutH* genes (Fig. 1), this result is consistent with the proposal that the HutP protein is involved in transcription antitermination at the stem-loop structure.

***hut* promoter transcription.** Primer extension analysis was used to determine *hutP* RNA levels present in cultures grown with either glucose or citrate as the carbon source. Densitometry scanning revealed that the relative abundance of *hutP* RNA in wild-type cultures grown with glucose was 6- to 18-fold lower than in wild-type cultures grown with citrate (Fig. 3). In cells from which the RNA was isolated, histidase activity was 18-fold lower in glucose-grown cells than it was in citrate-grown cells.

The level of *hutP* RNA in SF168R (*hutO*$_{CR2}$*4*) cells grown with either glucose or citrate was twofold higher than in wild-type cells grown on citrate (Fig. 3). This result indicates that the *hutO*$_{CR2}$*4* mutation relieves CR of *hut* transcription. In addition, it demonstrates that the start points of *hut* transcription are identical in wild-type and *hutO*$_{CR2}$*4* cells.

**Effects of *pai* and *hpr* mutations on *hut* expression.** *B. subtilis* strains with mutations in either the *hpr* or *pai* gene are resistant to glucose repression of sporulation (9, 25, 49). However, CR of histidase expression in exponentially growing cultures was not affected by mutations in either of these two genes (data not shown). These results suggest that CR of sporulation and CR of *hut* expression are mediated by different mechanisms. This is consistent with the previously reported observation that the *crsA* mutation relieves glucose repression of sporulation but not that of *hut* expression (4, 59).

**Effect of the *ccpA* mutation on *hut* expression.** CR of α-amylase, which has been studied in nutrient broth cultures supplemented with glucose, is partially relieved by the *ccpA* mutation (22). While examining the effect of the *ccpA* mutation on CR of *hut* expression, the *ccpA* mutant was found to have a growth defect in minimal media. The *ccpA* mutant strain was unable to grow in minimal medium containing only glucose and $NH_4^+$ as carbon and nitrogen sources. When the growth medium contained either a tricarboxylic acid cycle intermediate, e.g., malate or citrate, or an amino acid whose degradation produced intermediates of the tricarboxylic acid cycle, e.g., aspartate, glutamate, or glutamine, *ccpA* mutant cultures grew but at a slower rate than did wild-type cultures (Table 5 and data not shown). In transformation studies, the *ccpA*::Tn*917-lacZ* insertion was linked 100% with the growth defect on glucose-minimal medium (data not shown).

Growth of the *ccpA* mutant was also partially inhibited by histidine in glucose-minimal medium (data not shown). Since histidine inhibition of growth was also observed in CcpA$^-$ strains containing the Δ*hutPH*::*neo* mutation, degradation of histidine via the *hut* pathway is not responsible for this growth inhibition (data not shown). Although we have no explanation for this phenomenon, we note that strains containing the *cdh-3* mutation also have the same growth phenotype (13). Therefore, to examine the effect of the *ccpA* mutation on the CR of *hut* expression, the *ccpA*::Tn*917-lacZ* insertion was transferred to *hutC1* mutant strain SF168C, which does not require addition of histidine for induction of *hut* operon expression. Histidase levels were determined in extracts of SF168C and SF256C (*ccpA*::Tn*917-lacZ*) cells grown with a variety of carbon and nitrogen sources. Regardless of the growth medium, both the doubling time and levels of histidase present in the *ccpA* mutant cultures were higher than those of the isogeneic parental culture (Table 5).

## DISCUSSION

Analysis of *hut-lacZ* fusions has identified two operators which contribute to regulation of *hut* operon expression by CR. Two different models could explain how CR of the *hut* operon is mediated. In the first model, CR is mediated primarily by binding of the *hut* catabolite repressor protein to the *hutO*$_{CR2}$ site, where it acts as a transcriptional roadblock for RNA polymerase. This type of regulatory mechanism is not unprec-

TABLE 5. Histidase activity in extracts of wild-type and CcpA mutant strains

| Medium[a] | SF168C (*hutC1*) | | SF256C (*hutC1 ccpA*::Tn*917-lacZ*) | |
|---|---|---|---|---|
| | Doubling time (min) | Histidase sp act[b] (U/mg of protein) | Doubling time (min) | Histidase sp act[b] (U/mg of protein) |
| Glu + Gln | 50 | 6.6 ± 0.5 | 88 | 153 ± 5 |
| Glu + Glt + N | 60 | 5.4 ± 0.5 | 185 | 74 ± 31 |
| Mal + Gln | 90 | 10.3 ± 0.5 | 110 | 90 ± 21 |
| Cit + Gln | 100 | 94 ± 5.0 | 130 | 134 ± 5 |

[a] Cultures were grown in MOPS minimal medium containing the indicated carbon and nitrogen sources. Abbreviations: Glu, glucose; Gln, glutamine; Glt, glutamate; N, $NH_4Cl$; Mal, malate; Cit, citrate.
[b] See Table 2, footnote *b*.

TABLE 6. *Bacillus* sequences similar to the catabolite repression consensus

| Gene, operon, or promoter[a] | DNA sequence[b] | Location[c] | Reference |
|---|---|---|---|
| Consensus | TGWNANCGNTNWCA | | 60 |
| $hutO_{CR1}$ | GTTAATAGTTATCA | +2 to +15 | This work |
| $hutO_{CR2}$ | TGAAACCGCTTCCA | +203 to +216 | This work |
| *amyE* | TGTAAGCGTTAACA | −3 to +11 | 44 |
| *amyL* (*B. licheniformis*) | TGGAAGCGCTTGCA | +179 to +192 | 31, 66 |
| *amyL* (*B. licheniformis*) | TTTATACAATATCA | −15 to −2 | 31 |
| *amyL* (*B. licheniformis*) | TATCATATGTTTCA | −6 to +8 | 31 |
| *gnt* | TGAAAGCGGTACCA | +141 to +154 | 40, 41 |
| *gnt* | TGAAAGTGTTTGCA | −46 to −33 | 18, 60 |
| *gnt* | TATTCACGTTATCA | −23 to −10 | 18, 60 |
| *xyl* (*B. megaterium*) | TGAAAGCGCAAACA | +123 to +136 | 52, 53 |
| *xyl* (*B. megaterium*) | TGTATATGATGAGA | −15 to −2 | 52, 53 |
| *xyl* (*B. subtilis*) | TGGAAGCGTAAACA | +134 to +147 | 29 |
| *xyl* (*B. subtilis*) | TATTTAAGATTAAA | −14 to −1 | 20 |
| $P_{SPAC}$ | TGTGAGCGGATAAC | +4 to +17 | 64 |
| $P_{gcaD}$ | TGAAGTCTCCTTGA | −46 to −33 | 46 |
| $P_{gcaD}$ | TTCTATGGATAAAA | −6 to +8 | 46 |

[a] Genotype symbols are those of Anagnostopoulos et al. (1), except for $P_{SPAC}$ (64). The genes, operons, or promoters are from *B. subtilis* unless otherwise noted.
[b] The underlined nucleotides deviate from the corresponding positions in the consensus sequence.
[c] The indicated nucleotide position is relative to the transcription start site (+1).

edented; it has been reported that, in certain situations, the *E. coli lac* and *purR* repressors can block transcription elongation (10, 21).

In the second model, binding of the *hut* catabolite repressor protein at the $hutO_{CR2}$ operator would stabilize binding of other repressor molecules to the $hutO_{CR1}$ operator by a cooperative interaction that would require DNA looping. This type of cooperative interaction has been observed in other systems. The ability of the *lac* and λ repressors to bind to low-affinity operators can be enhanced by DNA looping with repressors bound at a second wild-type operator (24, 42). Although the $hutO_{CR1}$ operator does not appear to be a high-affinity catabolite repressor-binding site, it may play an important role in the regulation of *hut* expression because of its position immediately adjacent to the *hut* promoter. This model predicts that CR would regulate transcription initiation at the *hut* promoter. The results of the primer extension experiment suggest that *hut* expression is regulated primarily at the level of transcription initiation. However, we cannot rule out the possibility that the lower level of *hut* RNA present in glucose-grown cultures is due to reduced *hut* RNA stability.

The results of the experiments showing that β-galactosidase expression from the $P_{SPAC}$-$hutO_{CR2}$-*lacZ* and $P_{gcaD}$-$hutO_{CR2}$-*lacZ* fusions is regulated by CR suggest that $hutO_{CR2}$ is sufficient for repression and support the transcriptional roadblock model. Examination of the $P_{SPAC}$ and $P_{gcaD}$ promoter sequences revealed the presence of sequences that have weak similarity to the CR consensus sequence (Table 6). This raises the possibility that binding of the CR repressor protein to the low-affinity CR operator sites located within these promoters is enhanced when the $hutO_{CR2}$ site is placed downstream of the promoters because of cooperative interaction between the bound repressor proteins. Thus, no definitive conclusions regarding the mechanism mediating CR of *hut* expression can be drawn. However, it should be noted that the two models proposed for *hut* CR are not mutually exclusive. It has been reported that the $lacO_2$ operator, which lies downstream of the *lac* promoter, contributes to *lac* operon repression both by blocking transcription elongation and by strengthening repressor binding to the $lacO_1$ operator (16).

The organization and locations of the CR sites within the *hut* operon are similar to those described for several other *Bacillus* genes. Deletion analysis of the *B. subtilis gnt* operon has identified a region located downstream of the *gnt* promoter which is involved in mediating CR (40, 41). This region contains a sequence with significant similarity to the CR consensus sequence (Table 6). In addition, it has been noted previously that the *gnt* promoter contains two sequences that share homology with the CR consensus (60; Table 6). Expression of the *B. subtilis* and *B. megaterium xyl* operons is subject to CR (20, 52). In both of these operons, a *cis*-acting site required for CR of *xyl* expression is located downstream of the transcription start site within the *xylA* gene (29, 52; Table 6). The *xyl* promoters also contain sequences which have weak similarity to the CR consensus sequence (Table 6).

The *B. licheniformis* α-amylase gene, *amyL*, is regulated by CR when expressed in either *B. licheniformis* or *B. subtilis* (31). When the *amyL* promoter was replaced with random *B. subtilis* chromosomal DNA promoter fragments, *amyL* expression in *B. subtilis* was still subject to CR (31). Located within the *amyL* coding region is a sequence that is highly similar to the CR consensus sequence (Table 6). This sequence is presumably responsible for the promoter-independent CR of *amyL* expression observed in *B. subtilis*. In addition, the *amyL* promoter contains two overlapping sequences with similarity to the CR consensus (Table 6). Although it has not been shown that these putative sites within the *gnt*, *xyl*, and *amyL* promoters are involved in CR, their presence raises the possibility that DNA looping may also be involved in CR of these genes.

Site-directed mutagenesis of *amyO*, the operator which mediates CR of the *B. subtilis* α-amylase gene, *amyE*, was used to define the CR consensus sequence (60). The *amyO* site overlaps the *amyE* transcription start site (44). This suggests that binding of the catabolite repressor at the *amyO* site would prevent binding of RNA polymerase at the *amyE* promoter. Among the *Bacillus* genes which appear to be regulated by CR at sites which have similarity to the proposed CR consensus sequence, *amyE* is unique in that it is the only gene which does not contain a downstream CR site located within a coding sequence.

The *cis*-acting site which mediates CR of the *citB* gene does not have sequence similarity to the *amyO*-derived consensus sequence (17). Moreover, CR of the *citB* gene requires both a rapidly metabolizable carbon source and a source of 2-keto-

glutarate (51). Thus, the genetic and physiological evidence suggests that *citB* responds to carbon availability by a different regulatory system than does the *hut* operon. All known *cis*- and *trans*-acting mutations which relieve glucose repression of the *dciA* operon also relieve amino acid repression (57). In contrast, amino acid repression and carbon CR of the *hut* operon are mediated by independent regulatory systems (2, 63). This argues that different systems regulate the expression of the *hut* and *dciA* operons in response to carbon and amino acid availability.

*B. subtilis* mutants defective in carbon metabolism are often unable to exert CR. Mutations affecting the utilization of glucose, mannose, fructose, and glycerol have been shown to affect CR of inositol dehydrogenase and gluconate kinase expression (45). The pleiotropic *cdh-3* mutant has reduced levels of pyruvate and 2-ketoglutarate compared with wild-type cells and thus may be partially defective in glycolysis (13). Since the growth of a *ccpA* mutant is impaired on minimal media containing glucose as the carbon source, the inability of the *ccpA* mutation to repress *hut* expression may result from a defect in the metabolism of carbon compounds. Sequence analysis suggests that CcpA is a DNA-binding protein (22). However, it has been reported that the CcpA protein does not bind specifically to the *gnt* CR operator (19). Thus, it is possible that CcpA participates indirectly in the CR of some *B. subtilis* genes.

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