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Genetics

Site-directed mutagenesis of a catabolite repression operator sequence in *Bacillus subtilis*

(gene regulation/α-amylase/enzyme hyperproduction/transcriptional control/*Bacillus* promoter)

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ABSTRACT **Catabolite repression of the *Bacillus subtilis* α-amylase gene (*amyE*) involves an operator sequence located just downstream of the promoter (*amyR*), overlapping the transcription start site. Oligonucleotide site-directed mutagenesis of this sequence identified bases required for catabolite repression. Two mutations increased both the 2-fold symmetry of the operator and the repression ratio. Although many mutations reduced the repression ratio 3- to 11-fold, some also caused a 2-fold or greater increase in amylase production. Others caused hyperproduction without affecting catabolite repression. Homologous sequences in other catabolite-repressed *B. subtilis* promoters suggest a common regulatory site may be involved in catabolite repression.**

Enzymes involved in the metabolism of complex carbon and energy sources are unnecessary under conditions of abundant, readily metabolized alternatives such as glucose. The repression of these enzymes by glucose has been termed catabolite repression (1). In *Escherichia coli*, catabolite repression is mediated through the cAMP receptor protein (CRP, also called CAP, catabolite gene activator protein), which in the presence of cAMP binds specific DNA sites near promoters and activates transcription (for reviews, see refs. 2–4). Specific contacts are made between CRP, which functions as a dimer, and the CRP-binding sites on the DNA. The binding sites are roughly homologous and have a partial 2-fold symmetry in their consensus sequence (5–7). Recent evidence suggests that transcriptional activation is mediated by protein–protein interaction between CRP and RNA polymerase (8).

Like *E. coli*, *Bacillus subtilis* is subject to catabolite repression but by a different mechanism. cAMP cannot normally be found in *Bacillus* species (9, 10), except under conditions of oxygen limitation (11) and is, therefore, unlikely to be involved in catabolite repression in *B. subtilis*. Genes for degradative enzymes such as *sdh* (12), *gnt* (13), *citB* (14), *hut* (15), *sacC* (16), the β-glucanase gene (17), and *amyE* (ref. 18; for additional references, see ref. 19) are subject to catabolite repression, as is the complex developmental pathway of sporulation (20, 21), including *spoOA* (22). We have been studying catabolite repression of the starch degrading enzyme α-amylase in *B. subtilis*. Catabolite repression in this simple model system occurs at the level of transcription and is eliminated by a mutation 5 bases downstream of the transcription start site. This mutation is in an operator-like region having 2-fold symmetry and partial homology to the *lac* and *gal* operators (18). Deletion analysis eliminated the possibility that any other sequence in the promoter region plays a critical role in catabolite repression (23).

To thoroughly analyze the contribution of the operator to catabolite repression of α-amylase, we isolated and characterized three dozen additional mutations in this region, generated by oligonucleotide site-directed mutagenesis.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions. The bacterial strains used were *E. coli* strain TG1 (K-12, Δ[*lac-proAB*], *supE, thi, hsdD5,* [F′, *traD36, proA*$^+$*B*$^+$, *lacI*^{q}, *lacZΔM15*]), and *B. subtilis* strain BRE (*trpC2, lys-3, recE4, amyE*). The plasmids used were p5′αGR10 (24), pAMY10 (25), pARED, and pGEMR1F (23). Plasmid pARED is a derivative of pAMY10 from which the *Eco*RI fragment containing the 5′ end of the amylase structural gene and the *amyR* region has been deleted. The *E. coli* phagemid pGEMR1F (Fig. 1) contains the *Eco*RI fragment deleted in the generation of pARED. Subcloning the *Eco*RI fragment containing operator mutations from pGEMR1F back into pARED reconstitutes the amylase gene and creates a plasmid identical to pAMY10 except for the mutations. In a similar way, pAR1GR10 was created by subcloning this *Eco*RI fragment from p5′αB10, which contains the *gral0* mutation. The operator mutant plasmids were named pGEMR1M*x* or pAR1M*x*, where *x* equals the identification number of the mutant, as noted in Tables 1 and 2. All strains were grown as described (23). Antibiotic concentrations were 10 μg/ml for chloramphenicol and 50 μg/ml for ampicillin.

Enzymes, Reagents, and Amylase Assays. All chemicals and reagents were at least reagent grade and were purchased from Sigma. Restriction enzymes, *Eco*RI and *Hpa* I, and DNA ligase were purchased from Promega Biotec. Amylase assays were done as reported (24).

Plasmid Isolations. Plasmids were isolated from *E. coli* and *B. subtilis* as described (23). When plasmids were isolated from *E. coli* strain TG1 for use as sequencing templates, a modification of the alkaline lysis procedure of Maniatis *et al.* (26) was required to obtain readable sequences. The replacement of the phenol/chloroform extraction step by an acid/phenol extraction, followed by a chloroform:isoamyl alcohol (24:1, vol/vol) extraction substantially improved the quality of the plasmid DNA as a template for double-stranded DNA sequencing. To prepare acid/phenol, redistilled phenol was equilibrated with 50 mM sodium acetate (pH 4.0) until the pH of the phenol reached 4.0. Samples were kept cool (<5°C) during the acid/phenol extraction.

DNA Sequencing. Double-stranded plasmid DNA was used for all sequencing reactions that were performed with a Sequenase kit purchased from United States Biochemical following the manufacturer's instructions. The primer (5′-d[GACAC-TCCTTATTTGA]-3′) used was synthesized by the University of Wisconsin Biotechnology Center (Madison, WI).

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Abbreviation: CRP, cAMP receptor protein.

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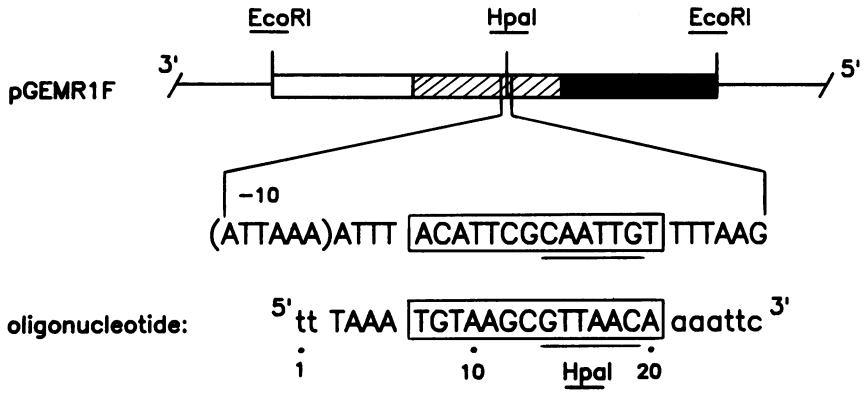

FIG. 1. Site-directed mutagenesis of the amylase operator region. The phagemid DNA included pGEM-3Zf(-) vector DNA (single line) a portion of the pAMY10 vector (open box), *amyR* region (hatched box), which includes the −10 region in parentheses, a portion of *amyE* (black box), and operator sequence (boxed bases). The *Hpa* I site in the operator is underlined, and the bases on the complimentary oligonucleotide used for mutagenesis are numbered. Only bases in the oligonucleotide with 2% degeneracy for other possible nucleotides (see *Materials and Methods*) are capitalized.

Oligonucleotide Site-Directed Mutagenesis. The mutagenesis of the operator region was performed by using the Amersham oligonucleotide-directed *in vitro* mutagenesis kit, according to the manufacturer's instructions. An oligonucleotide primer for the mutagenesis was prepared with 2% redundancy for each nonspecified base in positions 3–20 of the sequence 5'-d(TTTAAATGTAAGCGTTAACAAAATTC)-3' by the University of Wisconsin Biotechnology Center. This oligonucleotide mixture was hybridized to single-stranded DNA from the phagemid pGEMR1F containing the operator region as described (23).

Mutant Subcloning. Mutants were subcloned from pGEMR1*x* into pARED and transformed into *E. coli* and *B. subtilis* as described (23).

RESULTS

Isolation, Identification, and Subcloning of Mutants. Many of the oligonucleotide site-directed mutants were isolated by using a selection involving the *Hpa* I restriction site (see Fig. 1) present in the operator. The mutagenesis reaction produced double-stranded circular DNA that was subjected to *Hpa* I digestion before being used to transform *E. coli* strain TG1 to ampicillin resistance. Because plasmids that did not contain a mutation in this restriction site were linearized, the population of mutant transformants was greatly enriched. More than 65% of the transformants had plasmids resistant to *Hpa* I digestion and with the proper-sized insert DNA. Most remaining transformants had plasmids with deletions of the *amyR* region containing the *Hpa* I site, although a few transformants were still cleaved by *Hpa* I and contained the proper-sized insert. Sequence analysis confirmed that all proper-sized plasmids resistant to *Hpa* I cleavage contained at least one mutation in the operator *Hpa* I site. Most possible base changes in this site were isolated (Table 1).

Table 1. Effect of single-base substitutions on amylase expression

Mutant	Sequence	Amylase, % wild type*	Amylase repression ratio†
	↓		
wt	TAAATGTAAGCGTTAACA	100	14.5
2	A----------	195	16.5
144	-----A----------	129	6.3
217	----------T---------	246	2.8
GR10	----------A-------	246	2.4
30	----------T-------	125	2.1
25	----------G------	133	9.2
72	----------C------	138	28.8
1	----------G-----	157	4.0
36	----------C-----	238	3.8
81	----------G----	78	11.7
67	----------C----	101	13.0
4	----------T----	55	20.5
49	----------G---	157	4.0
46	----------T---	136	13.9
29	----------G-	175	2.4
38	----------T-	287	2.8

The operator sequence is boxed. wt, Wild type.
Transcription starts at the adenine marked by arrow.
*Relative amylase specific activity of nonrepressed cultures grown in nutrient sporulation medium to T_4 (time of maximal amylase accumulation), expressed as % of wild-type (*amyR1*) levels.
†Amylase repression ratio is the amylase-specific activity of a nonrepressed culture divided by the amylase-specific activity of an identical culture grown under repressing conditions. The ratio was determined from T_4 samples.

Table 2. Effect of multiple-base substitutions and single- or multiple-base deletions on amylase expression

Mutant	Sequence	Amylase, % wild type	Amylase repression ratio*
	↓		
wt	TAAATGTAAGCGTTAACA	100	14.5
amyR2*	A----------G-----	226	4.4
Double mutants			
3	A----------G-----	193	4.6
202	-----A---C----------	97	2.1
113	------C----T---------	135	1.6
147	------T---------T----	70	3.6
115	--------CG----------	11	1.7
192	--------C-----G------	9	1.9
410	---------G---A------	81	3.8
16	----------C------C---	42	2.2
70	----------A------T---	99	7.4
119	----------A-C------	290	2.6
45	----------G-----C---	80	2.7
211	----------A-------C	258	2.3
7	----------T-A-----	122	2.0
34	----------A-G----	54	5.0
48	----------A---T	246	2.2
3- and 4-base substitutions			
10	----------T--G-T-	157	1.5
222	-------C-G-GT-------	11	2.0
Deletions			
77	-(Δ)----------	159	40.2
109	-(Δ)---------A------	257	5.8
165	-----Δ--------(Δ)-T---	226	2.1
44	----------Δ--A-----	4	4.3
24	----------(Δ)-----	194	2.7
33	----------(Δ)---	213	3.2

Transcription starts at the adenine marked by arrow.
*From *B. subtilis natto*. This is not a mutant, but the sequence is different from *amyR1* [wild type (wt)] in two places in the mutagenized region.

Mutations in other bases were isolated by transforming *E. coli* TG1 with oligonucleotide-mutagenized DNA not pretreated with *Hpa* I and screening for mutants by sequence analysis. The frequency of mutations so isolated was <20%. Mutants 1, 2, and GR10 of Table 1 and mutant 3 of Table 2 have been described (18, 23) and were included in these tables for comparison.

Mutations were subcloned in pARED to make pAR1M*x* (see figure 9 in ref. 23). Plasmids were isolated from *E. coli* TG1 colonies that produced amylase and checked by restriction digestion before being used to transform *B. subtilis* strain BRE to amylase$^+$ and chloramphenicol resistance.

Effect of Single Base Substitutions on Repression. The amylase repression ratio is the amylase-specific activity of a broth culture grown under nonrepressing conditions (no glucose) divided by the amylase-specific activity of a glucose-grown culture. In wild-type *amyR1*, this ratio was between 14 and 15 and represented a 14- to 15-fold repression of amylase synthesis by glucose. The *B. subtilis* var. *natto* (*amyR2*) was only repressed 4- to 5-fold by glucose, but this allele caused >2-fold hyperproduction of amylase (Table 2, ref. 23).

We obtained single-base substitutions for 8 of the 14 bases in the operator (Table 1). In six of eight positions we obtained 2 or more different base substitutions. The resulting phenotypes indicated that position of the mutation was not the only important factor; different changes at the same position sometimes had different effects. Two specific changes, in particular, in mutants 4 and 72, actually increased the repression ratio, whereas other changes at the same position had little or no effect (Table 1). Both single-base changes in mutants 4 and 72 increased the 2-fold symmetry of the operator site by changing the right half-site, suggesting that the left half-site, TGTAAGC, is the optimal operator half-site sequence. Mutations in the cytosine at +4 (with respect to the +1 transcription start site adenine), guanine at +5, thymine at +7, and cytosine at +10 severely reduced the repression ratio. An A → G mutation at position +9 reduced the repression ratio, but an A → T mutation had no effect. Mutations of the thymine at +6 and the adenine at +8 had little or no effect on repression ratio.

Effect of Single-Base Substitutions on Hyperproduction. Amylase hyperproduction was caused by specific changes in the operator region and by one change 4 bases upstream of the operator region (23). Specific base changes at the +4 cytosine, +5 guanine (to adenine), +7 thymine (to cytosine), and +10 cytosine caused >2-fold increase in amylase production. Other base changes had less or no effects. These same bases were implicated in reducing the repression ratio.

Effect of Multiple Base Substitutions and Deletions on Repression. Most double mutations severely reduced the repression ratio, except those in mutants 3, 34, and 70 (Table 2). The least affected was mutant 70, which had changes at positions +3 and +9. Both changes were predicted to have little importance for operator function from evidence of single-base substitutions (Table 1). Mutant 16 had different base changes at the same positions but was no longer sensitive to catabolite repression.

Two double mutants with a +1 A → C change in common (mutants 115 and 192, Table 2) had a 10-fold reduction in amylase production and concomitant loss of catabolite repression. This mutation changed the transcription start site from adenine to cytosine, which is not normally used in *B. subtilis* for transcription initiation.

Several of the double mutants consisted of 1-base-pair (bp) changes examined as a single bp substitution (Table 1) and a second bp change not previously analyzed. Some previously examined substitutions caused relatively minor changes in the repression ratio; assuming that any change in repression ratio of the double mutants must be primarily from the second base change seemed reasonable. This assumption allowed us to deduce information about three positions for which no single-base change was available. Mutants 70, 147, and 192 (Table 2) each have a base change on the left half of the operator not represented in our collection of single-base changes and a second base change on the right half that either increased the repression ratio (mutant 147) or left it essentially unchanged (mutant 70, and to a lesser extent, mutant 192) as single-base substitutions (Table 1). If the final repression ratio is considered to be the product of the independent effects of each 2-bp change, we can refine our estimate of the direct contribution of these changes in the double mutants. We estimate the repression ratio for the G → T change in mutant 147 to be 2.6, the ratio for A → C in mutant 192 to be 3.0, and the repression ratio for G → A in mutant 72 to be 1.3. In addition, two other double mutants are amenable to this analysis, mutants 34 and 119. We estimated the C → A change in mutant 119 has an estimated repression ratio of 1.3 and the T → A change in mutant 34, an estimated ratio of 6.1.

Catabolite repression was eliminated in mutants having 3- and 4-base substitutions and for most deletion mutations; however, one deletion actually increased the repression ratio. This deletion lay outside the operator itself, but shifted the operator one base closer to the −10 region of the promoter. An identical deletion mutation accompanied by a T → A base change at position +6 (not represented in the collection of single-base substitutions) yielded a reduced repression ratio. One mutant, 44, in which the cytosine at position +4 was deleted and containing a base change at +7, reduced amylase production 20-fold.

Effect of Multiple Base Substitutions and Deletions on Hyperproduction. Only four of the multiple-base substitution mutants showed 2-fold or greater increase in amylase production (mutants 3, 48, 119, and 211). None of the 3- and 4-base substitutions significantly increased amylase production; in fact, 222 with 4-base substitutions had only 11% of the wild-type amylase level. On the other hand, most deletion mutations yielded 2-fold or greater amylase production, except mutant 44 discussed above, and mutant 77, which had greatly improved catabolite repression. All the multiple-base substitutions or deletions that showed hyperproduction of amylase had at least one mutation in a base identified by single-base substitutions as important for hyperproduction, except for mutant 109, for which no single-base mutation corresponding to the T → A change at position +6 was isolated.

DISCUSSION

Many single- and multiple-base substitutions in the amylase catabolite repression operator reduced the repression ratio. In analyzing repression ratios, we considered a ratio under 3 to be virtual derepression. A ratio between 3 and 6 was modest repression (this encompasses the repression ratio of the *amyR2* allele), and a repression ratio >6 was strong repression. A repression ratio of 6 means 6-fold repression.

The specific changes allowed us to deduce an operator consensus sequence: **TGWA*ANC ↓ GNTNWCA** where boldface letters represent the most critical bases, N is any base, W indicates adenine or thymine, the vertical arrow denotes axis of symmetry, and A* is the transcription start site (18).

Two mutations that increased the 2-fold symmetry of the operator by making the right half-site more complementary to the left increased operator strength. From this, we deduced an optimal operator of TGTAAGCGCTTACA. In this way, the amylase operator is analogous to the *gal* and *lac* operators in *E. coli*, to which it is highly homologous (18). Both of these operators also have optimal left half-sites, and increasing the symmetry of the right half-site with the left increased the

efficiency of repressor binding the operator (ref. 27, and S. Adhya, personal communication).

A single-base deletion that moved the operator closer to the −10 region of the promoter (mutant 77, Table 2) increased operator strength. If specific protein–protein interactions occur between the repressor molecule and polymerase, as has recently been proposed for CRP and polymerase in *E. coli* (8), this deletion mutation may strengthen that interaction. On the other hand, the catabolite repressor protein could mediate catabolite repression by interfering with polymerase in the recognition of, or binding to, the promoter or in the initiation of a transcriptionally active complex. Moving the binding site of a repressor protein 1/10 turn of the helix could enhance its ability to block transcription by any of these mechanisms.

The 2-fold symmetry of the operator site indicates that the catabolite repressor protein in *B. subtilis* is probably a dimeric DNA-binding protein having an α-helix-turn-α-helix DNA-binding domain (for reviews, see refs. 28–31). Other *Bacillus* operators have 2-fold symmetry and are recognized by repressor molecules having an α-helix-turn-α-helix DNA-binding domain. These include the *B. subtilis gnt* repressor, which binds an operator between −10 and +15 with respect to the start site of transcription. This region has perfect 2-fold symmetry with a half-site of ATACTTGTA (13). The *B. subtilis merR* gene product is a repressor and transcriptional activator of the *mer* (mercury resistance) operon. The operator has partial 2-fold symmetry between the −35 and −10 regions of the promoter and a consensus half-site of TACCCTGTAC. The repressor has a helix-turn-helix region near the amino terminus (32). In *B. licheniformis*, the production of penicillinase (*penP*) is negatively controlled by the repressor (*penI*), which binds at three operator sites with a consensus half-site of ANNMTTACAW (where M indicates adenine or cytosine). The *penI* gene product represses the expression of *penP* by physically blocking RNA polymerase binding (33).

The *gnt*, *mer*, and *penP* operators are probably not part of global regulatory systems like catabolite repression because no homologous sequences or similarly regulated genes have been described. On the other hand, the amylase catabolite repression operator shares significant homology with sequences in other *Bacillus* amylase gene regulatory regions, with other catabolite repressed genes, and with some genes for which the mode of regulation is unknown (Table 3). This homology varies, as it does for the 18 known CRP-binding sites in *E. coli* (3, 5). However, the similarities in the sequence and position of at least some of these potential

Table 3. Comparison of sequences near promoters of catabolite-repressed or potentially catabolite-repressed genes in *Bacilli*

Gene	Sequence	Reference
Bacillus amylase genes		
B. subtilis 168 *amyR1*	TAATTTTAAA T G T A A G C G T T A A C A AAATTCTCCA	18
B. subtilis natto amyR2	TAATTTAAAA T G T A A G C G T G A A C A AAATTCTCCA	34
B. subtilis strain DLG *amyR*-DLG	TAAGTTGAAA T G T A A G C G T T A A C A AAATTCTCCA	Unpublished†
B. polymyxa β-α-*amylase*	ATACTATTTT T G T A G A C G T T T A C A AAATGTTGTC	35, 36
B. stearothermophilus α-*amylase*	AATTCGATAT T G A A A A C G A T C A C A AATAAAAATT	37
B. licheniformis amyL	TGAGCGCAAA T A A C A G C G T C A G C A ATCGGGCGTA	38
B. amyloliquefaciens α-*amylase*	CTGATGTTTT T G T A A T C GgC A A A C T GACAAATAAC	39, 40
Other catabolite-repressed *Bacillus* genes		
β-glucanase	GAAAGAATCA T G T A A G A − T G A A C A TAGAAAACGC	17
spoOA	ATAGCGGTTT T G T CgA A T G T A A A C A TGTAGCAAGG	22
sacC	AACACAGTTG T G TtA A G C G T T T T C A TTGTTATTTC	16
gnt (−10)	ATAAAAGAAA T A T T C A C G T T A T C A TACTTGTATA	41, 42
gnt (−35)	AAATTAGAAA T G A A A G T G T T T G C A TAAAGAAAT	
sdh	TTATAATTTA T G T A C G C G T T T T C T TGACG CCCTT	12, 43
hut (upstream)	AAGGTTTTTT T A T A G T C T T T A A C A AGTTAGATTG	44
hut (downstream)	ATAATACTCA G T T A A T A G T T A T C A GAATTTTTAG	
Other *Bacillus* genes		
epr	ATCTTATTTT T G A A A A C G C T T A C A AAATTCATTT	45
B. natto senN	AGGTCGGTAT T G T A T G A A T T A A C A TGGTCAGTAC	46
dciAB	AATATAATTT T G T T A G A A T A T T C A TAATTTAGTA	Unpublished‡
B. brevis tycA	CTATAATGAG T T T C A G C G T C A G T A ACCTAGTGCT	47
B. amyloliquefaciens apr	CTGATGGAAA C G T A A G C G A A A T C A GTCTTTGGCT	48
Consensus	T G W N A N C G N T N W C A	
Assigned position	−7–6–5–4–3–2–1 1 2 3 4 5 6 7	

Sequence landmarks are identified as follows: a solid bar is shown over every known −10 or −35 promoter region. Potential −10 and −35 regions are designated by dots above these sequences. Possible transcription start sites are shown by a ?. A number over a sequence identifies the base directly under the last digit of the number. Dashes or lowercase letters represent gaps or bases not part of the consensus, respectively. The number of occurrences of bases in the consensus sequence in the 20 sequences listed is as follows: assigned position −7, 18; −6, 15; −5, 20; −4, − ; −3, 15; −2, − ; −1, 14; +1, 16; +2, −; +3, 12; +4, − ; +5, 17 ; +6, 19; +7, 18. W, adenine or thymine.

*Known transcription start site.

†P. King & G.H.C., unpublished data.

‡C. Mathiopoulos, J. Mueller, F. Slack, & A. L. Sonenshein (1989) Abstracts of the Fifth International Conference on Genetics and Biotechnology of *Bacilli*.

operators suggest that a common catabolite repression protein or a set of closely related proteins mediate catabolite repression in *B. subtilis*.

Our analyses indicate that some flexibility in the operator sequence is tolerated for catabolite repression function. The significance of the exact base differences between the sequences in Table 3 is not yet known. None of the specific differences were among the mutations generated in our studies. We also have not studied the effect of single-base insertions on the strength of this operator. Several alignments are optimized by a single-base insertion. We know that the operator is relatively intolerant to changes in spacing due to single-base deletions at several sites (Table 2).

We observed that hyperproduction and catabolite repression of amylase can both be affected by mutations in the same region and sometimes the same mutation. Several models can explain how this could occur. We favor one in which a catabolite repressor protein is responsible for repression, but hyperproduction is a function of the energy required for formation of a transcriptionally active complex. Changes in sequences downstream of the −10 region of a promoter can affect promoter strength by at least 10-fold (49, 50). Thus, mutations in the amylase operator region could affect catabolite repression and promoter strength simultaneously but by different mechanisms.

The similarity of the α-amylase catabolite repression operator to sequences in or near other catabolite-repressed *Bacillus* species promoters suggests that the amylase operator may be part of a global catabolite repression system. In this global system a repressor protein, activated by the presence of glucose or other readily metabolized carbohydrates in the growth medium, would bind to the operator-like sequences blocking transcription of the affected genes.

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