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Isolation and Characterization of a *cis*-Acting Mutation Conferring Catabolite Repression Resistance to α-Amylase Synthesis in *Bacillus subtilis*†

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***Bacillus subtilis* 168GR10 was shown to contain a mutation, *gra-10*, which allowed normal temporal activation of α-amylase synthesis in the presence of a concentration of glucose that is inhibitory to activation of amylase synthesis in the parent strain, 168. The *gra-10* mutation was mapped by phage PBS-1–mediated transduction and by transformation to a site between *lin-2* and *aroI906*, very tightly linked to *amyE*, the α-amylase structural gene. The *gra-10* mutation did not pleiotropically affect catabolite repression of sporulation or of the syntheses of extracellular proteases or RNase and was unable to confer glucose-resistance to the synthesis of chloramphenicol acetyltransferase encoded by the *cat-86* gene driven by the *amyE* promoter region (*amyR1*) inserted into the promoter-probe plasmid pPL603B. It therefore appears that *gra-10* defines a *cis*-regulatory site for catabolite repression, but not for temporal activation, of *amyE* expression. The evidence shows that temporal activation and glucose-mediated repression of α-amylase synthesis in *B. subtilis* 168 are distinct phenomena that can be separated by mutation.**

When faced with nutrient deprivation, the bacterium *Bacillus subtilis* activates a battery of genes normally silent during vegetative growth (22). One of these temporally regulated genes, *amyE*, encodes the extracellular enzyme α-amylase (α-1,4 glucan-4 glucanhydrolase; EC 3.2.1.1). No compound has been observed to trigger the activation of *amyE*, suggesting that the synthesis of α-amylase is not "inducible" in the classical sense; however, α-amylase synthesis is repressed by more readily metabolized carbon sources, such as glucose (13; reviewed in reference 23).

Based upon the above observations, a simple model explaining the regulation of α-amylase synthesis was proposed (28). In this model, the rate of α-amylase synthesis varies inversely with the intracellular concentration of a hypothetical carbon source catabolite; hence, both the activation and repression of α-amylase synthesis could be controlled in this model by a single mechanism, catabolite repression. If this hypothesis were correct, one would predict that a mutant no longer sensitive to glucose-mediated repression of α-amylase synthesis would produce α-amylase constitutively.

Here we report the isolation and characterization of a *cis*-acting mutation specifically conferring glucose insensitivity to α-amylase synthesis, and we present evidence that the activation and repression of α-amylase synthesis in *B. subtilis* are distinct phenomena that can be separated by mutation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The *B. subtilis* and *Escherichia coli* strains and plasmids used in this study are listed in Table 1. The following media were used: nutrient sporulation medium (30); Spizizen minimal medium (34) supplemented with 0.1% glucose and the appropriate auxotrophic requirements at 20 μg/ml; medium C (21) containing 0.1% sucrose or 0.5% glucose; A3 medium (antibiotic medium no. 3; Difco Laboratories, Detroit, Mich.); peptone-blood agar base (GIBCO Laboratories, Grand Island, N.Y.), and peptone-blood agar base to which 1.0% starch (potato starch; Sigma Chemical Co., St. Louis, Mo.) or 1.0% starch and 2.0% glucose had been added; and R protoplast regeneration medium (29) modified as described by Sanchez-Rivas

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype	Source (reference)
B. subtilis		
168	*trpC2*	Laboratory stock
168GR5	*trpC2 gra-5*	NTG[a] mutant of 168
168GR10	*trpC2 gra-10*	NTG mutant of 168
BRE	*lys-3 trpC2 recE4 amyE*	Laboratory stock
1A46	*thr-5 recE4 trpC2*	Bacillus Genetic Stock Center
1A289	*amyE aroI906 metB5 sacA321*	Bacillus Genetic Stock Center
WLN-7	*amyE aroI906 sacA321*	1A46$\xrightarrow{tf}$1A289; Met$^+$
WLN-9	*aroI906 sacA321*	1A46$\xrightarrow{tf}$1A289; Met$^+$/Amy$^+$ by congression
IS24	*lin-2 cysA14*	Issar Smith (11)
WLN-11	*gra-10 sacA321*	168GR10$\xrightarrow{tf}$WLN-7; Aro$^+$/glucose-resistant Amy$^+$
WLN-12	*gra-10 lin-2 sacA321*	IS24$\xrightarrow{tf}$WLN-11; Lm^r
E. coli		
HB101	F$^-$ *hsdS20 recA13 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44* λ$^-$	Laboratory stock (6)
Plasmids		
pAMY10	Cm^r *amyR1-amyE*$^+$ from 168	D. Henner (1, 41)
pPL603B	Km^r "promoterless" *cat-86*	D. Rothstein (37)
p5′αB10	Km^r Cm^r	This study

[a] NTG, *N*-Methyl-*N*′-nitro-*N*-nitrosoguanidine.

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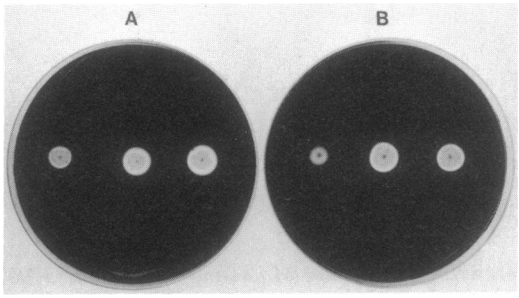

FIG. 1. Mutants of *B. subtilis* 168 resistant to repression of α-amylase synthesis by glucose. Colonies were incubated overnight and then flooded with I_2-KI solution as described in the text. Plates contained (from left to right) strains 168, 168GR5, and 168GR10 grown on (A) peptone-blood agar base containing 1.0% starch or (B) peptone-blood agar base containing 1.0% starch and 2.0% glucose.

(27). Davis salts used for dilutions consisted of 0.7% K_2HPO_4, 0.3% KH_2PO_4, 0.1% $(NH_4)_2SO_4$, and 0.005% $MgSO_4$. Kanamycin sulfate (Km) and chloramphenicol (Cm) were purchased from Sigma. Lincomycin (Lm) was a gift from The Upjohn Co., Kalamazoo, Mich. Selective antibiotic media contained 5 μg of kanamycin per ml (100 μg/ml in R plates), 10 μg of chloramphenicol per ml, or 100 μg of lincomycin per ml. Growth in liquid culture was monitored with a Klett-Summerson colorimeter fitted with the no. 66 red filter. All liquid cultures were incubated with vigorous aeration at 37°C.

DNA extraction. Extraction of chromosomal DNA from *B. subtilis* cultures, grown overnight in A3, was performed by a modification (14) of the method of Saito and Miura (25). DNA concentration was determined by the diphenylamine reaction (7). Plasmid DNA extraction from 1- or 2-liter *B. subtilis* cultures, grown overnight in A3 containing the appropriate antibiotic, was performed by a cleared lysate procedure (4), followed by ultracentrifugation in two successive CsCl-ethidium bromide gradients (24), four extractions with isoamyl alcohol, and extensive dialysis against 10 mM Tris-hydrochloride (ph 8.0)–1.0 mM disodium EDTA.

Transformation and transduction. Preparation of competent *B. subtilis* cells, preparation of phage PBS-1 generalized transducing lysates, and methods for transformation and transduction have been described previously (14). Transformation of *B. subtilis* protoplasts with plasmid DNA was performed by the method of Chang and Cohen (8). Regeneration of cells from protoplasts was accomplished on modified R solid medium (27, 29) containing the appropriate antibiotics.

Mutagenesis. Generation of mutants that were insensitive to glucose-mediated repression of α-amylase synthesis was performed by a modification of the method of Saito and Yamamoto (26). *B. subtilis* 168 was grown to midexponential phase (Klett reading of 50) in A3. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (Sigma) was added to 100 μg/ml (final concentration), and incubation was continued for 40 min. The cells were harvested by centrifugation, washed twice by suspension in Davis salts, and recultured in A3 with shaking. After culture lysis, the mutagenized culture was allowed to attain the midexponential phase once more. Appropriate dilutions of the culture were spread onto peptone-blood agar base containing 1.0% starch and 2.0% glucose. After overnight incubation, the plates were flooded with approximately 2 ml of a solution of 0.5% (wt/vol) I_2 and 5.0% (wt/vol) KI. Colonies exhibiting an unstained halo of starch hydrolysis were picked immediately, streak purified, and checked for the *trpC2* marker of the parental strain.

Enzymatic assays. α-Amylase activity from the culture supernatant of cells grown in liquid culture was quantitated by a modification of the procedure of Smith and Roe (33). A 1.0-ml sample of 50 mM Tris-hydrochloride (ph 6.8)–25 mM $CaCl_2 \cdot 2H_2O$–0.05% soluble starch was mixed on ice with 0.25 ml of culture supernatant, appropriately diluted in growth medium, and then incubated at 37°C for 30 min. The reaction was stopped by the addition of 0.5 ml of 0.01% (wt/vol) I_2–0.1% (wt/vol) KI in 1 N HCl, and the absorbance at 620 nm was compared with that of a blank treated identically, except without added α-amylase. Under these conditions, a decrease in absorbance at 620 nm of 0.1 was defined as 1 U of α-amylase activity, and the linear range of the assay was found to extend to a decrease of 0.6 absorbance unit.

Chloramphenicol acetyltransferase (CAT) activity was determined by the method of Shaw (31). The specific activity

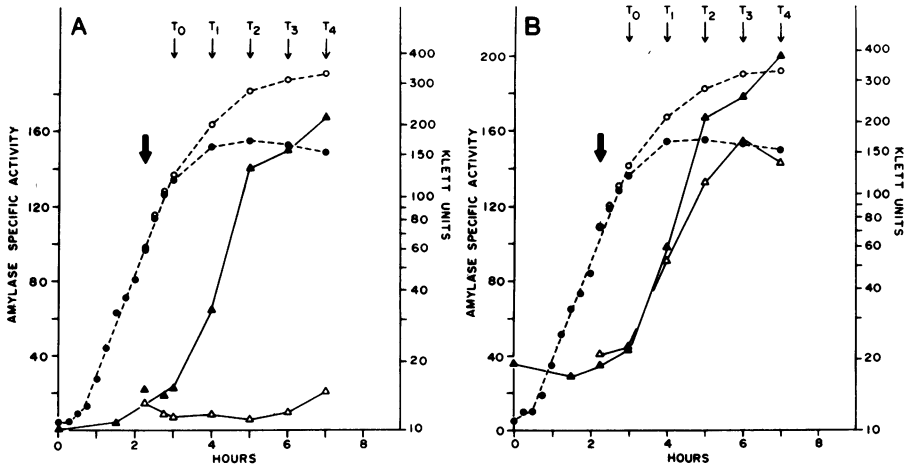

FIG. 2. Regulation of α-amylase synthesis (▲, △) during growth (●, ○) of strains 168 (A) and 168GR10 (B) in nutrient sporulation medium with (△, ○) or without (▲, ●) added glucose. At midexponential growth (heavy arrow), the culture was split, and one half received glucose to 1.0% final concentration. At the indicated times, 1.0-ml samples were removed from cultures, and the culture supernatants were assayed for α-amylase activity as described in the text. T_0, T_1, etc., denote hours after the end of exponential growth.

TABLE 2. Two-factor transformation crosses

Donor genotype (strain)	Recipient genotype (strain)	Selected phenotype	Recombinant classes[a]			No. of recombinants	% Linkage
			aroI	*amyE*	*gra-10*		
gra-10 (168GR10)	*aroI906 amyE* (WLN-7)	Aro$^+$	1	1		73	16.0
			1	0		383	
	aroI906 (WLN-9)	Aro$^+$	1		1	52	9.8
			1		0	480	

[a] Donor and recipient markers are indicated by 1 and 0, respectively.

of α-amylase and CAT was defined as units of enzymatic activity per milligram of cellular protein, as determined by the Lowry protein assay (17) with bovine serum albumin as a standard.

Restriction endonucleases and T4 DNA ligase were purchased from Promega-Biotec or from New England Biolabs and were used in accordance with the manufacturers' recommendations. Plasmid DNA, restriction endonuclease cleavage products, and ligation products were routinely analyzed after electrophoresis in 1.0% agarose gels cast and run in 89 mM Tris–89 mM boric acid–2 mM disodium EDTA containing 0.1 μg of ethidium bromide per ml. Restriction endonuclease-generated DNA fragments were purified from preparative agarose gels by direct electroelution (38) into DEAE-nitrocellulose paper (no. NA-45; Schleicher & Schuell Co., Keene, N.H.).

RESULTS

Regulation of α-amylase synthesis in strain 168GR10. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine–induced mutants of *B. subtilis* 168 capable of producing α-amylase in the presence of an otherwise repressing concentration of glucose were isolated (Fig. 1). One of these mutants, designated strain 168GR10, was chosen for further examination. When grown in liquid culture, either nutrient sporulation medium (Fig. 2B) or C medium containing glucose as the sole carbon source (data not shown), strain 168GR10 was observed to activate α-amylase synthesis at the end of exponential growth, regardless of the presence or absence of glucose in the medium. Under identical conditions, α-amylase synthesis in strain 168 was severely repressed by glucose addition (Fig. 2A). The temporal activation of α-amylase synthesis at the end of exponential growth in strain 168GR10 was similar to that seen in strain 168, indicating that the mechanism controlling the activation of α-amylase synthesis was not altered by mutation.

Transductional and transformational analyses of *gra-10*. Preliminary data indicated that the mutation in 168GR10 responsible for glucose-resistant α-amylase synthesis (*gra-10*) was linked by transformation to *aroI906*, an auxotrophic marker in the region of the *B. subtilis* chromosome near *amyE*, the structural gene for α-amylase (Table 2). Previous workers have mapped *amyE* and a tightly linked locus controlling the rate of α-amylase production, *amyR1*, to the region of the *B. subtilis* chromosome between the markers *lin-2* and *aroI* (for reviews, see references 15 and 23). We therefore constructed a strain containing the *lin-2* and *gra-10* markers in an *amyE*$^+$ *aroI*$^+$ genetic background (strain WLN-12; Table 1) to use as a donor in three-factor crosses in this region, using the generalized transducing phage PBS-1. The results of two crosses, mapping both *amyE* and *gra10* between *lin-2* and *aroI906*, are presented in Table 3. The maps generated from these crosses are shown in Fig. 3. The three-factor transduction data, in conjunction with the two-factor transformation data, suggest that *gra-10* is less tightly linked to *aroI* than is *amyE*. This roughly locates *gra-10* on the upstream side of *amyE*, the approximate location of the rate-controlling locus, *amyR1*.

Regulation of α-amylase synthesis encoded by a cloned *amyE* gene. Several groups have reported cloning α-amylase genes from various bacilli (10, 16, 19, 20, 32, 36, 42), including *B. subtilis* (35, 41). The wild-type *amyE*$^+$ gene cloned from *B. subtilis* 168 was reported to be expressed in either *B. subtilis* or *E. coli* (41), suggesting that the cloned DNA fragment may also contain the *amyR1* DNA region required for the regulation of *amyE* expression. We obtained

TABLE 3. Three-factor transduction crosses

Donor genotype (strain)	Recipient genotype (strain)	Selected phenotype	Recombinant classes[a]				No. of recombinants	Probable order
			aroI	*amyE*	*gra-10*	*lin-2*		
lin-2 gra-10 (WLN-12)	*aroI906 amyE* (WLN-7)	Aro$^+$	1	1		1	669	*aroI amyE lin-2*
			1	1		0	200	
			1	0		1	6	
			1	0		0	113	
		Lm^r	1	1		1	48	*lin-2 amyE aroI*
			0	1		1	9	
			1	0		1	7	
			0	0		1	12	
	aroI906 (WLN-9)	Aro$^+$	1		1	1	492	*aro-I gra-10 lin-2*
			1		1	0	178	
			1		0	1	2	
			1		0	0	76	
		Lm^r	1		1	1	130	*lin-2 gra-10 aroI*
			0		1	1	8	
			1		0	1	0	
			0		0	1	44	

[a] Donor and recipient markers are indicated by 1 and 0, respectively.

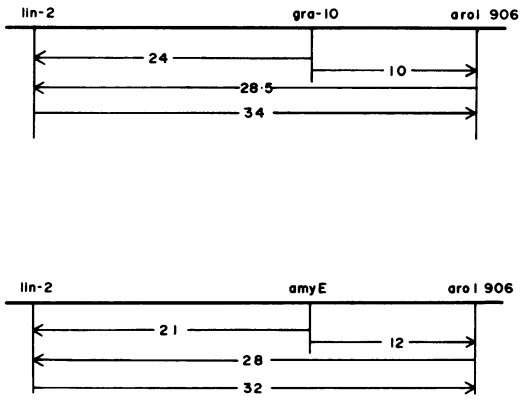

FIG. 3. Transduction map of the locations of the *amyE* and *gra-10* mutations by phage PBS-1-mediated transduction. Numbers are 100 minus percent cotransduction. Arrows point to selected markers.

the cloned *B. subtilis* 168 *amyE*$^+$ gene (41) inserted into the *E. coli-B. subtilis* shuttle plasmid pBS42 (1). The resultant plasmid, designated pAMY10, can be maintained in either *B. subtilis* or *E. coli* and confers a Cm^r, Amy$^+$ phenotype to either host (D. Henner, personal communication). When propagated in *B. subtilis* BRE (*recE4 amyE*$^-$), the *amyE* gene carried on pAMY10 was temporally activated and was repressed by glucose (Fig. 4) in a manner directly analogous to the regulation of chromosomally encoded α-amylase synthesis in strain 168 (Fig. 2A), although at an elevated level, presumably due to the increased dosage of the plasmid-borne *amyE* gene. When pAMY10 was propagated in *E. coli* HB101, α-amylase synthesis was constitutive and not repressed by glucose (data not shown), which is consistent with the findings of other workers that the catabolic repression system of *B. subtilis* is not mechanistically analogous to the cyclic AMP-mediated system found in *E. coli* (3, 5, 9, 12).

Location of the regulatory DNA controlling cloned *amyE* expression. The finding that the cloned *amyE* was regulated normally in *B. subtilis* indicated that the *B. subtilis* DNA fragment in pAMY10 also contained the *amyR1* regulatory DNA sequences necessary for properly timed activation and for glucose-mediated repression of α-amylase synthesis. The location of this regulatory DNA on the 5′ end of the *amyE*-bearing insert from pAMY10 was confirmed by the construction of a plasmid in which the 1.2-kilobase-pair *Eco*RI fragment from pAMY10, consisting of *amyE* DNA coding for the NH_2 terminus of α-amylase plus 400 base pairs of upstream DNA, was inserted into the promoter-probe plasmid pPL603B (37; Fig. 5). When the fusion plasmid, designated p5′αB10, was introduced into *B. subtilis* strain BRE, CAT synthesis from the *cat-86* gene was regulated in a fashion parallel to both plasmid-borne and chromosomally encoded α-amylase synthesis, both in terms of temporal activation and of glucose-mediated repression (Fig. 6).

***gra-10* mutation exerts its effect in *cis*.** Based upon the observations of close genetic linkage between *gra-10* and *amyE*, and the pattern of α-amylase regulation in strain 168GR10, it seemed possible that *gra-10* could be a mutation either in a *cis*-acting regulatory site or in a locus, tightly linked to *amyE*, encoding a *trans*-acting regulatory factor. To distinguish between these possibilities, plasmid p5′αB10 was introduced into strains 168 and 168GR10, creating strains in which the synthesis of two distinct enzymatic activities, chromosomally encoded extracellular α-amylase and plasmid-encoded intracellular CAT, were both under the control of the regulatory DNA derived from the 5′ end of the *amyE* gene. If *gra-10* were to affect a *trans*-acting regulatory factor, both α-amylase and CAT synthesis in strain 168GR10(p5′αB10) should be resistant to repression by glucose. Alternatively, if *gra-10* were to affect a *cis*-acting regulatory site, only chromosomally encoded α-amylase expression should be glucose resistant in strain 168GR10(p5′αB10). Both α-amylase and CAT activity were temporally activated and glucose repressible in the control experiment performed on strain 168(p5′αB10) (Fig. 7). In strain 168GR10(p5′αB10) (Fig. 8), plasmid-encoded CAT activity was repressed by glucose, even though the simultaneous synthesis of α-amylase was resistant to glucose-mediated repression, indicating that in the *trans* configuration the *gra-10* mutation was unable to overcome catabolite repres-

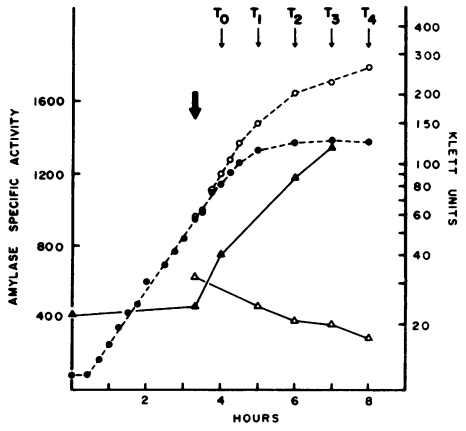

FIG. 4. Regulation of plasmid-borne α-amylase synthesis (▲, △) during growth (●, ○) of strain BRE(pAMY10). Cells were grown in nutrient sporulation medium containing 10 μg of chloramphenicol per ml and were treated as described in the legend to Fig. 2.

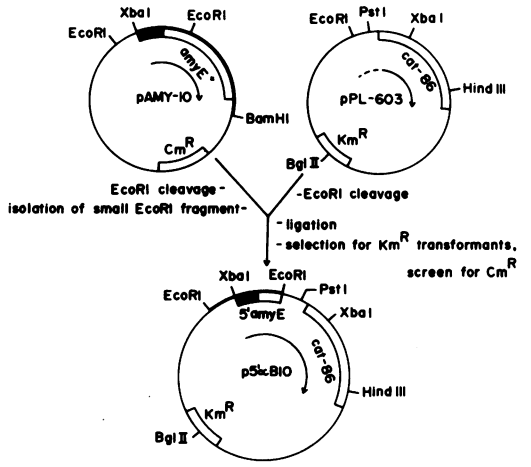

FIG. 5. Construction of p5′αB10. Plasmid pAMY10 was cleaved with *Eco*RI, and the 1.2-kilobase-pair fragment was purified from a preparative agarose gel as described in the text. The purified *amyE* promoter-containing fragment was mixed with *Eco*RI-cleaved pPL603B and ligated. The ligation mixture was introduced into protoplasts of strain BRE as described in the text. Plasmid sizes: pAMY10, 8.3 kilobase pairs; pPL603B, 5 kilobase pairs; p5′ αB10, 6.2 kilobase pairs.

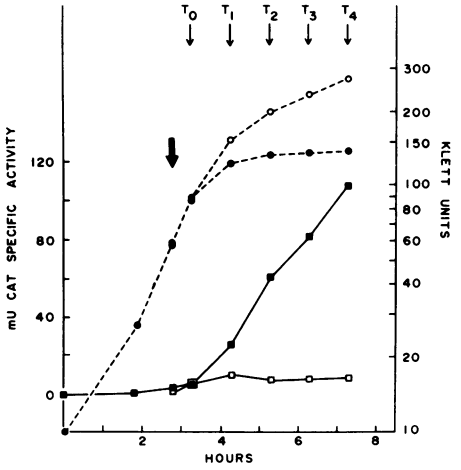

FIG. 6. Regulation of CAT synthesis (■, □) during growth (●, ○) of strain BRE(p5'αB10). Cultures were grown in nutrient sporulation medium containing 5 μg of kanamycin per ml and were treated as described in the legend to Fig. 2. At the indicated times, 1.0-ml samples were harvested by centrifugation, suspended in 0.1 ml of medium, and assayed for CAT activity (31).

sion for CAT synthesis. Apparently, the *amyE* regulatory DNA contained in plasmid p5'αB10 does not encode a *trans*-acting product capable of restoring glucose sensitivity to chromosomally encoded α-amylase activity in strain 168GR10 (Fig. 8A). This evidence suggests that the *gra-10* mutation is *cis* dominant for the resistance to catabolite repression of *amyE* expression in strain 168GR10.

Effect of *gra-10* on other catabolite repressible events. The *gra-10* mutation apparently confers glucose resistance upon α-amylase synthesis specifically. Preliminary experiments indicate that the production of extracellular proteases and RNase, events whose activation are temporally coincident with that of α-amylase (22, 28), as well as the ability of cells to sporulate, were repressed by glucose in strain 168GR10 in a manner identical to the repression of these events in strain 168 (data not shown).

DISCUSSION

The regulation of α-amylase synthesis in *B. subtilis* has been well characterized at the phenomenological level (23, 28), but much less so at the genetic and molecular levels. It has been shown that α-amylase synthesis repressed by easily metabolized carbon sources, such as glucose, but not by nitrogen sources, such as NH_3 (13). The existence of a locus controlling the rate of α-amylase synthesis, tightly linked to the *amyE* structural gene, was first reported by Yuki (44) and was proposed to be the promoter region for the α-amylase gene (43, 44). This locus, designated *amyR1* in *B. subtilis* 168, exists in at least two other allelic forms; *amyR2*, derived from *B. subtilis* subsp. *natto* (44), and *amyR3*, derived from *B. subtilis* subsp. *amylosaccharitícus* (43). Both the *amyR2* and *amyR3* alleles have been introduced into *B. subtilis* 168-derived strains by DNA-mediated transformation and are reported to confer an amylase hyperproducing phenotype upon transformants (43, 44). In addition to the reported α-amylase hyperproductivity, we have observed that the *amyR2* locus also confers resistance to glucose-mediated repression of α-amylase synthesis, whereas temporal activation of *amyE* expression is unaffected (unpublished data).

The nucleotide sequences of the *amyR1* and *amyR2* loci have been determined (40, 41). We have compared the two sequences and found extensive stretches of homology; however, there are sequence differences between the two loci, particularly in an A+T-rich region upstream from the putative promoter sequences. Similar A+T-rich stretches have been observed in close proximity to promoters for developmentally regulated genes in bacilli (18, 39) and are reported to affect promoter utilization in *B. subtilis* (2, 18).

Based upon the evidence presented in this paper that the *gra-10* mutation (i) specifically confers resistance to catabolite repression of α-amylase synthesis, (ii) exerts its effect in *cis*, and (iii) is tightly linked to the *amyE* structural gene, we propose that *gra-10* is an additional allele of the *amyR* regulatory locus, possibly defining a target site involved in the catabolic repression of *amyE* expression. The fact that *gra-10* and *amyR2* do not affect the timing of *amyE* activation strongly suggests that the activation of α-amylase syn-

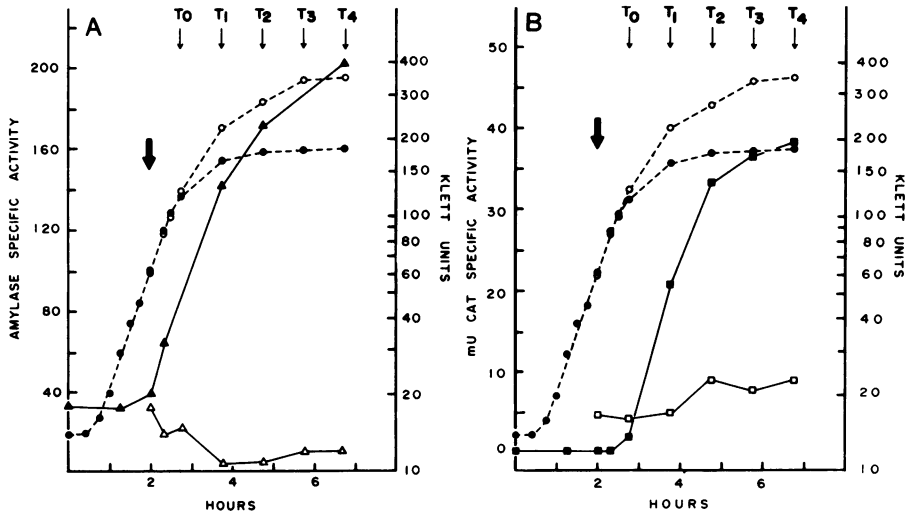

FIG. 7. Simultaneous regulation of α-amylase (A; ▲, △) and CAT (B; ■, □) synthesis during growth (●, ○) of strain 168(p5'αB10). Cultures were grown in nutrient sporulation medium containing 5 μg of kanamycin per ml and were treated as described in the legends to Fig. 2 and 6.

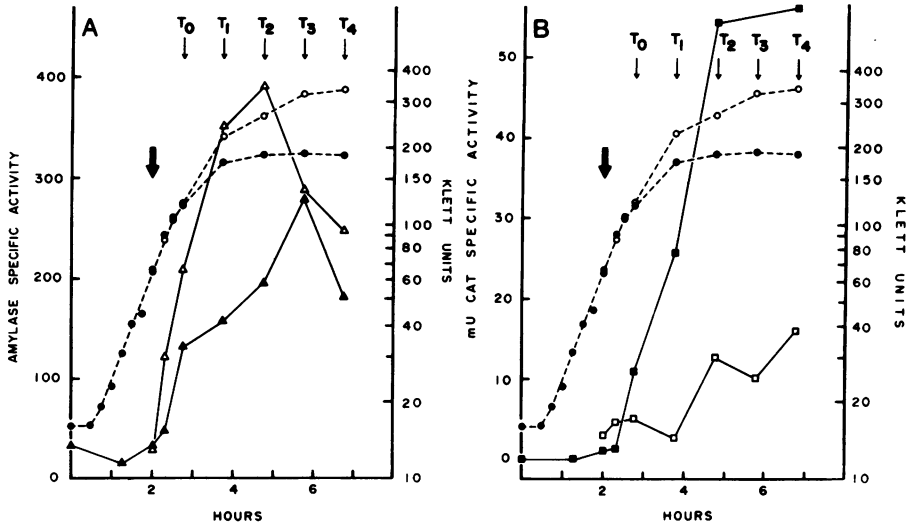

FIG. 8. Simultaneous regulation of α-amylase (A; ▲, △) and CAT (B; ■, □) synthesis during growth (●, ○) of strain 168GR10(p5′αB10) in nutrient sporulation medium containing 5 μg of kanamycin per ml. Cultures and samples were treated and assayed as described in the legends to Fig. 2 and 6.

thesis is controlled by a mechanism other than catabolite repression, in contrast to previously proposed models (23, 28).

At the moment, a simple explanation of the glucose-resistant character of α-amylase synthesis observed in strain 168GR10 is that the *gra-10* mutation affects a regulatory DNA sequence upstream from the *amyE* gene which is recognized by a (hypothetical) *trans*-acting regulatory molecule responsible for repression of *amyE* expression in the presence of an excess of readily metabolized carbon sources. Alternatively, it is possible that mutation at the *gra-10* locus has resulted in the creation of a new promoter sequence that is insensitive to catabolite repression for amylase expression. Evidence indicating that the temporal activation of α-amylase synthesis is not altered in strain 168GR10 renders this possibility less tenable, however. Experiments are currently in progress to clone the regulatory DNA region containing the *gra-10* mutation and to determine its nucleotide sequence, to further characterize this operator-like region of DNA at the molecular level.

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