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Genetic Analysis of the Promoter Region of the *Bacillus subtilis* α-Amylase Gene†

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The *amyR2* allele of the *Bacillus subtilis* α-amylase *cis*-regulatory region enhances production of amylase and transcription of *amyE*, the structural gene, by two- to threefold over *amyR1*. The amylase gene bearing each of these alleles was cloned on plasmids of about 10 to 15 copies per chromosome. Transcription of the cloned amylase gene by each *amyR* allele was activated at the end of exponential growth and was subject to catabolite repression by glucose. The amount of amylase produced was roughly proportional to the copy number of the plasmid, and cells containing the *amyR2*-bearing plasmid, pAR2, produced two- to threefold more amylase than cells with the *amyR1* plasmid, pAMY10. Deletion of DNA 5′ to the α-amylase promoter, including deletion of the A+T-rich inverted repeat found in *amyR1* and *amyR2*, had no effect on expression or transcription of α-amylase. Deletion of DNA 3′ to the *amyR1* promoter did not impair temporal activation of chloramphenicol acetyltransferase in *amyR1–cat-86* transcriptional fusions, but catabolite repression was abolished. When an 8-base-pair linker was inserted in pAMY10 at the same site from which the 3′ deletion was made, amylase expression doubled and was repressed less by glucose. Both the deletion and the insertion disrupted four bases at the 3′ end of the putative amylase operator region. Site-directed mutagenesis was used to change bases in the promoter-operator region of *amyR1* to their *amyR2* counterparts. Either change alone increased amylase production twofold, but only the change at +7, next to the linker insertion of 3′ deletion site, yielded the increased amylase activity in the presence of glucose that is characteristic of the *amyR2* strain. The double mutant behaved most like strains carrying the *amyR2* allele.

The expression of *Bacillus subtilis* α-amylase involves activation of synthesis at the end of exponential growth (30) and repression by easily metabolized carbon sources (11, 36; our unpublished results). This resembles regulation of the sporulation process (for a review, see reference 20). The catabolite control mechanisms governing α-amylase synthesis and sporulation differ at least partially (27), but the mechanism for temporal activation may be the same.

The *B. subtilis* α-amylase gene, *amyE*, is regulated by the *cis*-acting region *amyR* (44, 49). There are at least three alleles of the *amyR* locus: *amyR1* (from *B. subtilis* 168), *amyR2* (from *B. subtilis* subsp. *natto*), and *amyR3* (from *B. subtilis* subsp. *amylosaccharitiicus*) (48). *amyR2* and *amyR3* are amylase-hyperproducing alleles of *amyR1* (44, 48, 49). *amyR1* and *amyR2* have been cloned and sequenced (45, 46). Regulation of *amyR1* and *amyR2* occurs at the level of transcription. S1 nuclease mapping showed that transcription initiates at a site 8 base pairs (bp) downstream of a sequence 50% homologous to the promoter consensus recognized by $E\sigma^A$ (σ^{43}) RNA polymerase. This promoter was also used in vitro by purified *B. subtilis* $E\sigma^A$ RNA polymerase in runoff transcription experiments with either *amyR1* or *amyR2* DNA (29).

In strains containing either of these alleles, *amyE* mRNA rapidly accumulated after the onset of stationary phase, but was repressed when cells were grown in the presence of glucose (4, 29). The sequences for *amyR1* and *amyR2* have only two base differences in the promoter region but are strikingly different 5′ to the promoter. The *amyR2* sequence contains a 49-base inverted repeat in the A+T-rich upstream region. This has a potential free energy for RNA secondary structure of −15.4 kcal/mol, more than twice the potential free energy (−7.3 kcal/mol) of the 29-base A+T-rich inverted repeat sequence found in *amyR1*. It has been suggested that the *amyR2* inverted repeat is responsible for the hyperproduction phenotype (45). Deletion of this upstream palindromic sequence in an *amyR2-bla* fusion strain was reported to cause lower β-lactamase production and reduced glucose catabolite repression (39).

The *B. amyloliquefaciens* α-amylase regulatory region contains a 43-base A+T-rich inverted repeat upstream of a σ^A-type promoter (16). Deletion of the palindromic sequence had no effect on α-amylase regulation when cloned in *B. subtilis* (16). Instead, the inverted repeat functioned as a transcription termination signal for a 2.2-kilobase (kb) operon located upstream of the α-amylase gene (17).

In this communication, we demonstrate that the inverted repeat in both *amyR1* and *amyR2* is dispensable for α-amylase regulation in *B. subtilis*. Additionally, deletion of sequences 3′ to the promoter in *amyR1–cat-86* transcription fusions eliminated catabolite repression of chloramphenicol acetyltransferase (CAT) but had no effect on temporal activation at the onset of stationary phase. The two base differences between *amyR1* and *amyR2* immediately 3′ to the promoter are shown to contribute to the hyperproduction, and one of them, at +7, is responsible for the weaker catabolite repression that characterizes *amyR2*-directed amylase production.

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. *B. subtilis* strains were grown in NSM (nutrient sporulation medium [35]) for sporulation and amylase assays, A3 medium (antibiotic medium no. 3; Difco Laboratories, Detroit, Mich.) for plasmid isolation, or PBAB

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype	Source or reference
B. subtilis		
168	*trpC2*	Laboratory stock
BRE	*trpC2 recE4 lys-3 amyR1*$^+$ *amyE*	Laboratory stock
WLN-4	*sacA321 amyR1*	29
WLN-15	*sacA321 amyR2*	26
E. coli		
JM83	*ara* Δ(*lac-proAB*) *rpsL thi* ϕ80 *lacZ*ΔM15	47
JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*) (F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*ΔM15)	47
TG1	K12, Δ(*lac-proAB*) *supE thi hsdD5* (F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*ΔM15)	Amersham Corp.
Plasmids		
pAMY10	Cm^r *amyR1-amyE*$^+$	D. Henner (2, 46) (Fig. 1)
pEATα1W15	Em^r *amyR2-amyE*$^+$	26 (Fig. 1)
pAR2	Cm^r *amyR2-amyE*$^+$	This study (Fig. 1)
pARI7	Cm^r *amyE*$^+$	This study
pARIE	Cm^r *amyE*$^+$	This study
pAR2E	Cm^r *amyE*$^+$	This study (Fig. 4)
pAR105	Cm^r *amyE*$^+$	This study (Fig. 4)
pAR108	Cm^r *amyE*$^+$	This study (Fig. 4)
pAR202	Cm^r *amyE*$^+$	This study (Fig. 4)
pAR2110	Cm^r *amyE*$^+$	This study (Fig. 4)
pGEM-3Zf(−)	Ap^r *lacZ*$^+$	Promega
pGEM-3Zf(+)	Ap^r *lacZ*$^+$	Promega (Fig. 9)
pGEMR1	Ap^r *amyR1 lacZ*	This study
pGEMR108	Ap^r Δ*amyR1 lacZ*	This study
pGEMR110	Ap^r Δ*amyR1 lacZ*	This study
pPL603B	Km^r promoterless *cat-86*	D. Rothstein (43)
p5′αB10	Km^r *amyR1–cat-86*	25 (Fig. 7)
p5′αGR10	Km^r *gra-10–cat-86*	25 (Fig. 7)
pPLR18	Km^r Δ*amyR1–cat-86*	This study (Fig. 7)
pUC18	Ap^r *lacZ*$^+$	47
pMWR1	Ap^r *amyR1 lacZ*	29
pMWR2	Ap^r *amyR2 lacZ*	This study
pMWR2E	Ap^r Δ*amyR2 lacZ*	This study
pGEMR1F	Ap^r *amyR1 lacZ*	This study (Fig. 9)
pGEMR1M1	Ap^r *amyR1M1 lacZ*	This study (Fig. 9)
pGEMR1M2	Ap^r *amyR1M2 lacZ*	This study (Fig. 9)
pGEMR1M3	Ap^r *amyR1M3 lacZ*	This study (Fig. 9)
pAR1M1	Cm^r *amyR1M1 amyE*$^+$	This study (Fig. 9)
pAR1M2	Cm^r *amyR1M2 amyE*$^+$	This study (Fig. 9)
pAR1M3	Cm^r *amyR1M3 amyE*$^+$	This study (Fig. 9)
pARED	Cm^r Δ*amyR1 amyE*	This study (Fig. 9)

(peptone blood agar base; GIBCO Laboratories, Grand Island, N.Y.) for plating transformants. *E. coli* cultures were grown in LB medium (Luria-Bertani medium [22]). Liquid cultures were aerated by vigorous shaking and incubated at 37°C. Growth in liquid culture was monitored with a Klett-Summerson colorimeter with a no. 66 red filter.

Reagents, enzymes, and linkers. Restriction endonucleases and other DNA-modifying enzymes were purchased from Promega Biotech (Madison, Wis.) except for mung bean nuclease, which was a gift from Epicentre Technologies (Madison, Wis.). All enzymes and reagents were used according to the manufacturer's recommendations. All chemicals and antibiotics were reagent grade and were purchased from Sigma Chemical Co. (St. Louis, Mo.). Low-melting-point agarose was electrophoresis grade and obtained from Bethesda Research Laboratories, Gaithersburg, Md. Double-stranded phosphorylated linkers containing a *Hin*dIII (5′-pd[CAAGCTTG]-3′) or an *Eco*RI (5′-pd[GGAATTCC]-3′) restriction site were purchased from Pharmacia LKB Biotechnology Inc., Piscataway, N.J.

Plasmid isolation methods. Plasmids were isolated from 1.5 ml of *E. coli* broth cultures by the alkaline lysis method (22). *B. subtilis* plasmid DNA was isolated from 5-ml broth cultures by the rapid boiling method (14). Large amounts of purified plasmid DNA were prepared by alkaline lysis of 500- to 1,000-ml cultures, followed by CsCl-ethidium bromide density gradient ultracentrifugation (22).

Copy number determinations. Plasmid copy number was determined essentially as described by Weisblum et al. (42). *B. subtilis* plasmid-containing strains were grown in NSM with 50,000 cpm of [*methyl*-^{3}H]thymidine and vigorous aeration. Samples (1.5 ml) were removed, and crude lysates were prepared as described before (42). Ethanol precipitates were dried, suspended in TE (22), and subjected to electrophoresis on low-melting-point (12 by 10 cm) agarose slab gels cast and run in Tris-acetate electrophoresis buffer (22). Agarose gel slices containing DNA bands corresponding to plasmid and chromosomal DNA were separated and melted in 1 ml of H_2O; then, 9 ml of BioSafe scintillation fluid (Research Products International Corp., Mount Prospect, Ill.) was added to each sample, and the radioactivity associated with each band was quantitated by liquid scintillation counting. Background counts from degraded DNA were quantitated by preparing strain BRE without a plasmid in an identical manner. The chromosomal band was cut from the gel, as was a piece from where the plasmid would ordinarily be located in plasmid-containing strains. The ratio of background cpm to chromosome cpm was subtracted from ratios for plasmid-containing strains.

Exonuclease III deletions. Plasmids pAMY10 and pAR2 were linearized at their unique *Xba*I site and digested with exonuclease III by using the Erase-a-Base System purchased from Promega. The reactions were performed as described by the manufacturer except that exonuclease III digestion was carried out at 30°C with only 100 to 200 U of exonuclease III. Samples were withdrawn every 20 s for further treatment as recommended.

Plasmid transformation methods. *E. coli* cells were transformed as described by Hanahan (10). *B. subtilis* cells, made competent as described previously (26), were transformed with plasmid DNA.

DNA sequencing. Plasmid sequencing was performed with a Sequenase kit purchased from United States Biochemical Corp. (Cleveland, Ohio) following the manufacturer's instructions. The *amyR1 Hpa*I-*Eco*RI fragment, as well as two *amyR1* deletion *Hpa*I-*Eco*RI fragments, were subcloned into *Sma*I-*Eco*RI-cleaved pGEM-3Zf(−). These plasmids, pGEMR1, pGEMR108, and pGEMR110, were sequenced by using the vector SP6 primer as described by the manufacturer (Promega). The *amyR2* region, the remaining deletions, and the mutants carrying site-directed mutations were sequenced from the original plasmids by using a sequencing primer (5′-d[GACACTCCTTATTTGA]-3′) synthesized for us by the University of Wisconsin Biotechnology Center (Madison, Wis.).

Transcription mapping. RNA was isolated as described previously (29). *Pst*I-*Sin*I restriction fragments containing the *amyR1* or *amyR2* region were isolated from pMWR1 and pMWR2, respectively. These fragments were labeled as described previously (29) and used to identify *amyE*-specific

transcripts. Mung bean nuclease mapping was performed as described for S1 nuclease mapping (29) with several modifications. Ten volumes of mung bean nuclease buffer (30 mM sodium acetate, 50 mM NaCl, 1 mM zinc acetate, 5% [vol/vol] glycerol, pH 4.6), with 4 U of mung bean nuclease per ml, was added to the probe-RNA hybrid, and the mixture was placed on ice for 10 min. The samples were then placed at 37°C for 30 to 60 min, and the reaction was stopped by the addition of 1/8 volume of 1.5 M sodium acetate (pH 7.5). The probe-RNA hybrids were precipitated by adding 2 volumes of cold absolute ethanol, dried, and treated as described previously (29).

Enzyme assays. Assays of extracellular α-amylase and intracellular CAT activities were performed as described previously (25, 37).

Site-directed mutagenesis. Single-stranded DNA was isolated from *E. coli* JM109 or TG1 containing pGEMR1F or pGEMR1M2 by using phage R408 and the manufacturer's suggested procedures (pGEM single-strand system; Promega). The oligonucleotides containing the desired changes were synthesized for us by the University of Wisconsin Biotechnology Center: for M1, 5′-d(GTAAGCGTGAA CAA)-3′; for M2, 5′-d(GATAATTTAAAATGTA)-3′. Site-directed mutagenesis with the single-stranded DNA (ssDNA) and oligonucleotides listed above was performed with the Amersham oligonucleotide-directed in vitro mutagenesis system version 2 kit (Amersham Corp., Arlington Heights, Ill.) according to the manufacturer's instructions. Since a phagemid (pGEM) was being used, however, we isolated transformant colonies instead of plaques as described in the kit instructions.

RESULTS

Subcloning the *amyR2* allele. To conveniently study *amyR2*-directed amylase production and transcription of *amyE*, *amyR2* was subcloned into several plasmids used in previous studies of *amyR1*. Gene conversion transformation was used to clone *amyR2* from *B. subtilis* subsp. *natto* 1A412 (NA64) onto an Em^r plasmid to make pEATα1W15 (26). An *Eco*RI fragment from this plasmid, carrying the *amyR2* locus and part of the *amyE* gene, was ligated into pAMY10, replacing the analogous *amyR1*-bearing fragment, to create pAR2 (Fig. 1). The unique *Hpa*I site in pAMY10 is not present in the *amyR2*-containing plasmid pAR2, which had instead an extra *Ava*I site not found in pAMY10 (Fig. 1).

To generate a hybridization probe for transcription mapping, the *Xba*I-*Eco*RI restriction fragment from pAR2, which contains *amyR2* and part of the *amyE* gene, was subcloned into *Xba*I-*Eco*RI-digested pUC18. The resulting plasmid, pMWR2, was identical, except in the *amyR* region, to pMWR1, which we used in our previous transcription studies (29).

Comparison of *amyR1*- and *amyR2*-directed amylase production. We compared amylase production in two isogenic strains differing only in their *amyR* region. The *amyR2*-containing strain, WLN-15, consistently produced at least 2.5-fold more α-amylase than *amyR1*-containing WLN-4 (Fig. 2A). Amylase production in both strains was activated at the end of the exponential growth phase (T_0) and repressed in the presence of glucose. The basal level of amylase produced during exponential growth and the repressed levels in the presence of glucose were also at least 2.5-fold higher in WLN-15 than in WLN-4 during comparable growth conditions and times (Fig. 2A).

Multicopy plasmids pAMY10 and pAR2, containing the *amyE* gene and *amyR1* or *amyR2*, respectively, were introduced into a *B. subtilis* Amy$^-$ strain, BRE, by transformation. Amylase production was 2.5-fold higher in cells containing pAR2 than in cells containing pAMY10 (Fig. 2B). Temporal activation of amylase still occurred at T_0, and catabolite repression reduced amylase specific activity levels.

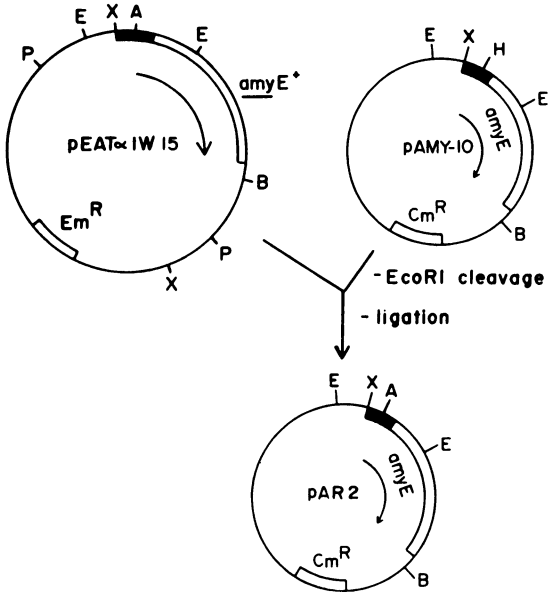

FIG. 1. Construction of pAR2. An *Eco*RI fragment from plasmid pEATα1W15 (25), carrying the *amyR2* locus and part of the *amyE* gene, was ligated into pAMY10 linearized with *Eco*RI. *E. coli* JM109 was transformed to Cm^r Amy$^+$ as described in Materials and Methods. Plasmids were isolated and screened by restriction digests for the replacement of the pAMY10 *amyR1* allele by *amyR2*. Plasmid sizes: pAMY10 and pAR2, 8.3 kbp; pEATα1W15, 11.3 kbp. A, *Ava*I; B, *Bam*HI; E, *Eco*RI; H, *Hpa*I; P, *Pst*I; X, *Xba*I; Em^r, erythromycin resistance determinant; Cm^r, chloramphenicol resistance gene. *amyE* is the structural gene for α-amylase, and the black box is *amyR*.

Effect of gene dosage on amylase production in strains containing plasmids. At all growth stages and times, amylase was present at about 10- to 18-fold greater amounts in strains with amylase plasmids than in corresponding *amyE*-single-chromosomal-copy strains WLN-4 and WLN-15 (Fig. 2A and B). We determined the plasmid copy number in *B. subtilis* BRE containing pAMY10, pAR2, pAR108, pAR105, pARI7, pAR202, pAR2E, and pAR2110. Copy number was estimated for exponentially growing cells (mid-log phase) and cells 2 h after the onset of stationary phase (T_2). Background counts for both sample times were estimated from DNA preparations of the plasmidless BRE strain. The counts in gel slices from the region corresponding to the plasmid location were used to determine the portion of counts per minute derived from sheared or degraded chromosomal DNA. The background ratio was substantially higher at T_2 (0.0268 versus 0.0101 at mid-log phase), at which time nuclease expression was substantially higher than at mid-log phase. The background ratio was subtracted from the plasmid ratio before copy number was calculated.

Between 7 and 16 plasmids were present per genome in BRE cells (Table 2). Overall, the plasmid copy number averaged 10 copies in mid-log phase and 11 in stationary phase (T_2).

Because glucose repressed amylase production, we examined its effect on plasmid copy number. Glucose, to a 1%

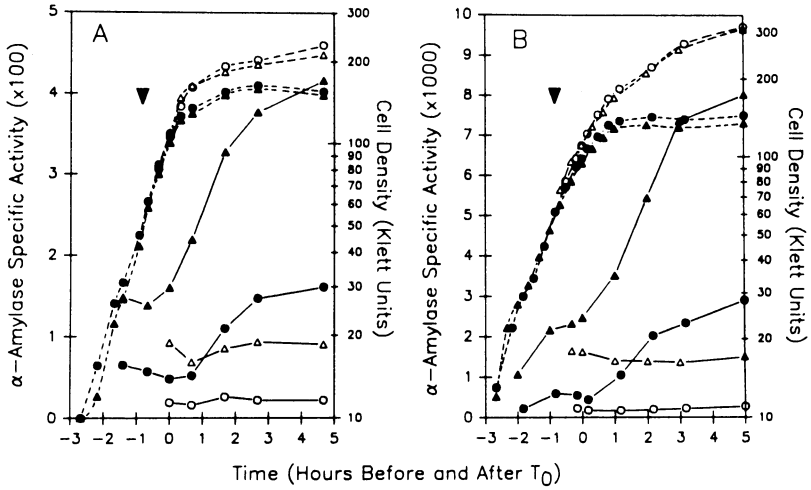

FIG. 2. Regulation of α-amylase synthesis (solid lines) during growth (dashed lines) of *B. subtilis* strains bearing *amyR1* (circles) or *amyR2* (triangles). Cells were grown in NSM with (open symbols) or without (solid symbols) glucose added at mid-log phase (solid arrowhead) to a 1% final concentration. (A) Amylase synthesis in WLN-4 (*amyR1*) and WLN-15 (*amyR2*) strains bearing single-copy chromosomal *amyR* alleles. (B) Amylase synthesis in strain BRE containing plasmid pAMY10 (*amyR1*) or pAR2 (*amyR2*). Amylase specific activity was defined as units of enzymatic activity (25) per milligram of protein. The growth stage is hours before and after T_0.

final concentration, was added to half of an exponentially growing culture immediately after the first sample (for copy number determination) had been withdrawn. BRE containing pAMY10 had an average of 15 copies at T_2 when grown in glucose and 13 copies in the absence of glucose. pARI7 was present at T_2 in 9 copies in the presence of glucose and 12 copies in its absence (data not shown).

Upstream deletions in pAMY10 and pAR2. DNA was deleted from the 5′ end of *amyR* in plasmids pAMY10 and pAR2 by digestion of *Xba*I-cleaved plasmids by exonuclease III. Digestion proceeded in both directions after linearization at the unique *Xba*I site of each plasmid (Fig. 1). Plasmids were recircularized and transformed into *E. coli*, selecting for Cm^r and screening for amylase production. Eighty-four of the 211 pAMY10 deletion Cm^r transformants produced amylase, as did 47 of 159 pAR2 deletion Cm^r transformants. The sizes of the deletions were determined by restriction digestion and agarose gel electrophoresis for 30 of the pAMY10- and 11 of the pAR2-derived deletion plasmids. Between 100 and 730 bp were deleted from the plasmids. Seventeen deletion plasmids, 12 from pAMY10 and 5 from pAR2, were transformed into *B. subtilis* BRE to characterize their amylase production. All 17 transformed Amy^- BRE to Amy^+. In all strains, amylase production was catabolite repressed by glucose. Only one deletion plasmid, pAR2110, produced low levels of α-amylase (Fig. 3B, inset); otherwise, the level was approximately equal to that produced by strains bearing the undeleted parent plasmid pAMY10 or pAR2 (data not shown). All plasmids, including pAR2110, increased amylase production near the beginning of stationary phase. Time courses of amylase production by strains containing sequenced deletion plasmids and corresponding undeleted controls are shown in Fig. 3. Two pAMY10 and two pAR2 derivatives had deletions into or beyond the inverted repeats (Fig. 4).

TABLE 2. Plasmid copy number at mid-log phase and T_2 growth stages

Plasmid	Growth stage	Plasmid/chromosome cpm ratio[a]	Estimated plasmid copy no.[b]
pAMY10	Mid-log	0.0320	15
	T_2	0.0224	10
pAR105	Mid-log	0.0147	7
	T_2	0.0170	8
pAR108	Mid-log	0.0203	10
	T_2	0.0159	8
pARI7	Mid-log	0.0267	12
	T_2	0.0274	12
pAR2	Mid-log	0.0212	10
	T_2	0.0169	8
pAR202	Mid-log	0.0200	10
	T_2	0.0326	16
pAR2E	Mid-log	0.0202	10
	T_2	0.0318	16
pAR2110	Mid-log	0.0215	10
	T_2	0.0197	9

[a] Background ratio was subtracted (see Results).

[b] The molecular masses in daltons (Da) used to calculate estimated plasmid copy number were 2.5×10^9 Da for the *B. subtilis* chromosome; 5.5×10^6 Da for pAMY10, pARI7, and pAR2; 5.2×10^6 Da for pAR105, pAR108, and pAR202; 5.2×10^6 Da for pAR2110; and 5.1×10^6 Da for pAR2E.

Since amylase production in strain BRE containing *amyR2*-derived plasmid pAR2110 was reduced to a level comparable to that of a strain with a single chromosomal copy, we cured the strain of the Cm^r determinant and screened for concomitant loss of amylase production. A broth culture of A3 medium, without antibiotic selection, was inoculated with a 3-week-old, fully sporulated colony from an NSM plate. The culture was grown overnight at 46°C, and cells were plated on PBAB plates. Of the 906 colonies screened by picking to PBAB supplemented with 1% starch or 1% starch and chloramphenicol (10 μg/ml), 47 were resistant to chloramphenicol and produced amylase. The remaining 859 were Cm^s and Amy^-, indicating that 94.8% of the cells were cured of the plasmid and that gene conversion of the *amyE* mutant chromosomal allele had not occurred. This indicated that the low level of amylase in BRE(pAR2110) was produced from the plasmid.

A deletion mutant of pAR2 analogous to the deleted *amyR2*–β-lactamase gene (*bla*) fusion of Takano et al. (39) was constructed. Plasmid pMWR2 was digested at the *Ava*I

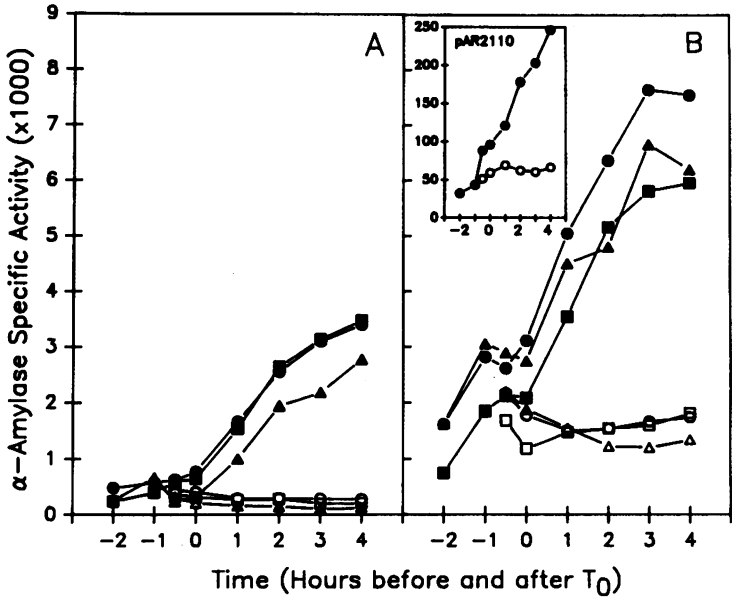

FIG. 3. Regulation of α-amylase synthesis during growth of *B. subtilis* BRE containing plasmids derived from *amyR1* (A) or *amyR2* (B) plasmids. Cells were grown as described in the legend to Fig. 2. Amylase activity in the absence of glucose is represented by solid symbols and in the presence of glucose by open symbols. Time is as described in the legend to Fig. 2. The plasmids are as follows: (A) pAMY10, circles; pAR105, triangles; pAR108, squares; (B) pAR2, circles; pAR202, triangles; pAR2E, squares. Inset: pAR2110.

site, dephosphorylated, and recircularized by ligation with phosphorylated *Eco*RI linkers. An *Eco*RI fragment, containing the *amyR2* promoter but not the inverted repeat, was ligated into *Eco*RI-digested pAMY10, replacing the *amyR1* *Eco*RI fragment and creating pAR2E (Fig. 4). Amylase production in pAR2E was temporally activated and repressed by glucose in a manner virtually identical to that in wild-type pAR2 (Fig. 3B).

Analysis of *amyE* transcription from plasmids. To ascertain whether the deletions affected transcription of amylase mRNA, we determined the 5′ *amyE* transcription start site in strains containing the deleted plasmids. RNA was isolated from cells grown in the presence or absence of glucose to T_2, the time at which amylase mRNA is being transcribed at its maximal rate (4, 29). An *amyR1* or *amyR2* probe was hybridized to equal amounts of RNA from cells containing

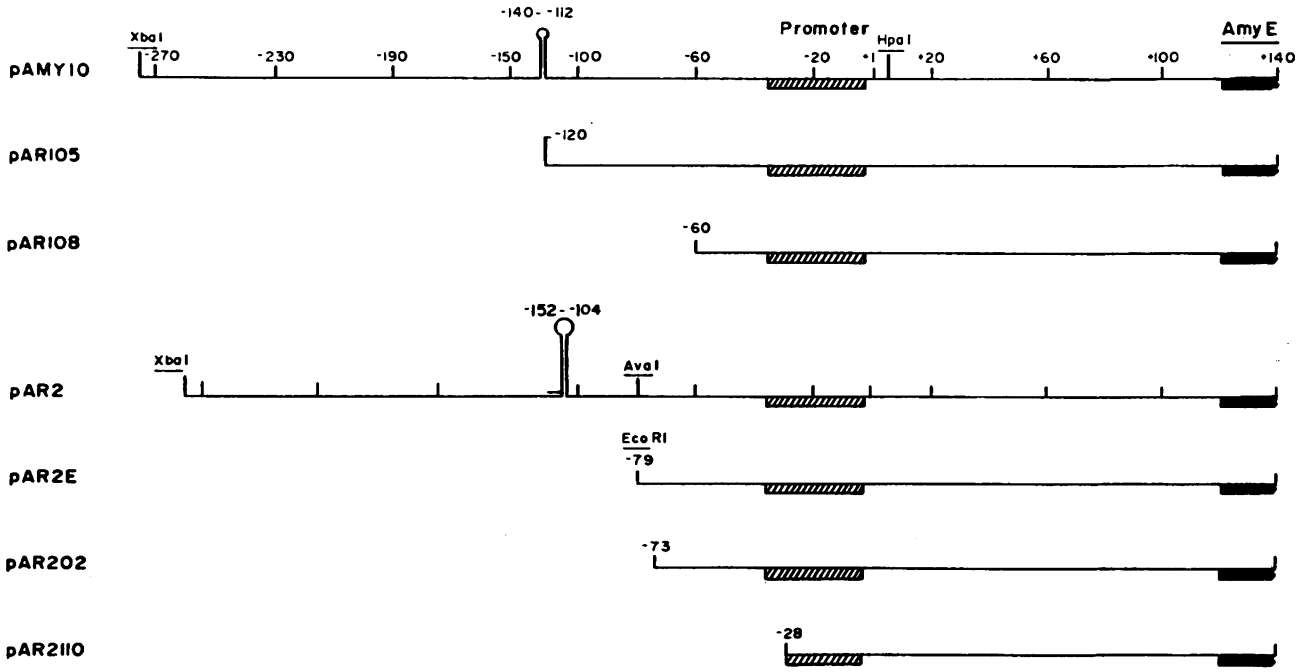

FIG. 4. Endpoints of upstream deletions in *amyR1* and *amyR2*. Endpoints were determined by sequencing as described in Materials and Methods. Numbers represent the distance (in base pairs) 5′ (−) or 3′ (+) from the transcription start site. The start of the *amyE* gene is shown as a solid bar; the promoter region is shown as a crosshatched bar.

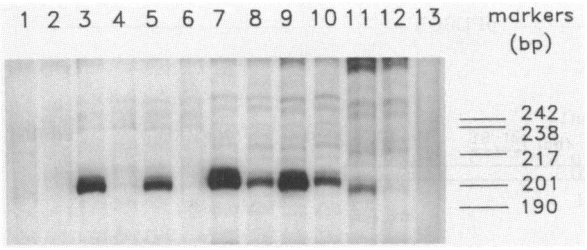

FIG. 5. Mapping the 5′ end of the *amyE* transcript. Mung bean nuclease-resistant DNA-RNA hybrids were analyzed on a 6% sequencing gel. Cellular RNA (20 μg) was used in reactions 3 to 10, and 100 μg was used in lanes 1, 2, 11, and 12. RNA was isolated from cells grown in NSM in the absence (lanes 1, 3, 5, 7, 9, and 11) or presence (lanes 2, 4, 6, 8, 10, and 12) of 1% glucose. RNA was isolated from strain BRE with no plasmid (lanes 1 and 2), pAMY10 (3 and 4), pAR108 (5 and 6), pAR2 (7 and 8), pAR202 (9 and 10), or pAR2110 (11 and 12). Lane 13 is the tRNA carrier control. Markers were end-labeled *Hpa*II fragments of plasmid pAT153, and sizes are indicated on the right.

the corresponding pAMY10 or pAR2 plasmid or deletion derivative. After digestion of remaining ssDNA or RNA by mung bean nuclease, the hybrid was resolved on 6% acrylamide sequencing gels (Fig. 5). We estimated the relative amounts of transcript produced. Transcription started at the same site in the deletion mutants as it did in pAMY10 and pAR2, the wild-type plasmids (Fig. 5). The same relative mRNA levels were also observed. The size of the hybrid resolved on the gels indicated that P1 was the promoter used, as it is in the chromosomal single-copy strains (4, 29) (Fig. 6). There was also about threefold more mRNA from cells containing pAR2 or pAR202 than from those containing pAMY10 or pAR108. Glucose repressed transcription to a level proportional to the amylase activity seen in cells at the analogous time.

Cells containing plasmid pAR2110, with a deletion extending through the −35 region of the promoter to −27, showed reduced transcription, as indicated by the fact that 5 times as much RNA was required to detect the transcript (Fig. 5, lane 11). This transcript was barely detectable when the cells were grown in medium containing 1% glucose (Fig. 5, lane 12). It appears to have a slightly greater mobility, indicating that it may initiate from P2, a potential promoter overlapping P1 (Fig. 6). Alternatively, it was noticed that all the faint degradation bands were also shifted in the gel to about the same extent; thus, the shift in the transcript band may be due to loading fivefold more RNA in those lanes and not to a new transcription start site. In any case, transcription was reduced, corresponding to lower amylase production, and was also repressed by glucose.

Deletion of DNA 3′ to the *amyR1* promoter. Because DNA upstream (5′) of the promoter did not seem to contribute to transcriptional regulation of α-amylase, we examined the effect on regulation of a deletion 3′ to the promoter. Because a downstream deletion could affect the amylase structural gene, we cloned an *amyR1* 3′ deletion into pPL603B, which contains a promoterless *cat* gene (43). We had previously shown that when the *amyR1* and *gra-10* regions were cloned into this plasmid, they regulated CAT production in a manner analogous to their regulation of α-amylase (25, 26).

The deletion was constructed by digesting pAMY10 with *Hpa*I, which cuts once at the unique site (5′-GTTAAC-3′) located 7 bp 3′ to the transcription start site (Fig. 6 and 7a). The linearized plasmid was dephosphorylated and recircularized by ligation with phosphorylated *Eco*RI linkers to make plasmid pARIE. The *Eco*RI fragment containing the promoter was removed and ligated into *Eco*RI-digested pPL603B to make pPLR18 (Fig. 7a).

The deletion of DNA 3′ to the *Hpa*I site did not affect temporal activation of *cat* but eliminated catabolite repression of CAT synthesis when cells were grown in the presence of glucose (Fig. 7b). This release from catabolite repression was analogous to that of p5′αGR10, in which *cat* expression is under the control of the *gra-10* mutation, which confers catabolite repression resistance to *amyR1* (26) (Fig. 7bC). The deletion eliminates the last four bases on the 3′ end of the putative amylase operator identified, in part, by the *gra-10* mutation (29).

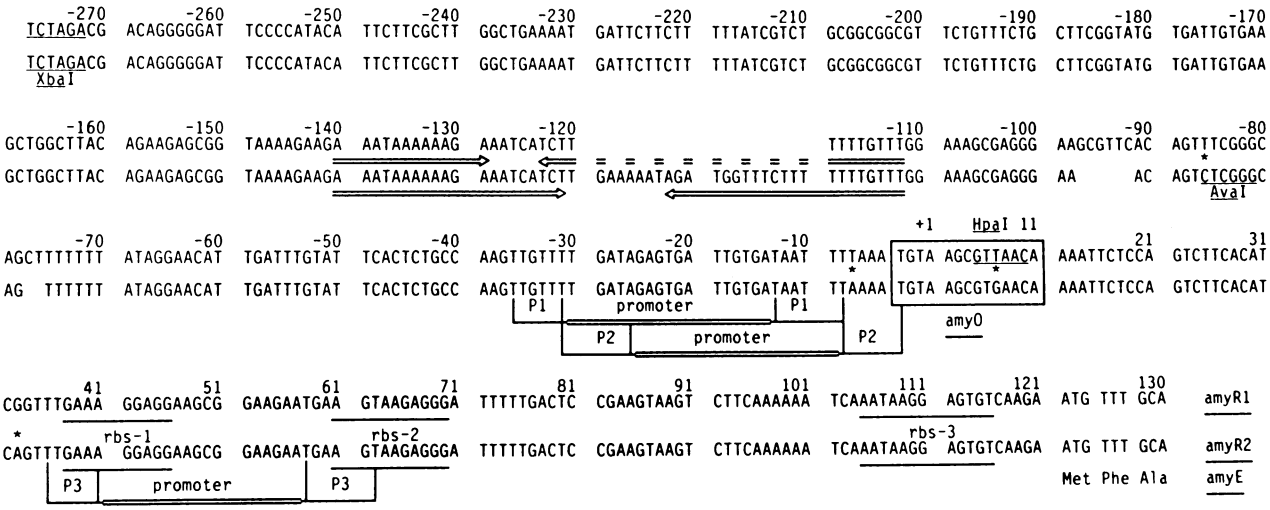

FIG. 6. Comparison of the *amyR1* and *amyR2* nucleotide sequences. The top sequence is *amyR1* and the bottom is *amyR2*; P1, P2, and P3 are σ^A-type promoter consensus sequences, of which P1 is utilized. rbs-1, -2, and -3 are putative ribosome-binding sites; stars (★) indicate base mismatches; the *Ava*I, *Hpa*I, and *Xba*I restriction sites are underlined; arrows indicate the inverted repeat sequences; the operator site (*amyO*) is enclosed in a box; and *amyE* is the amylase structural gene.

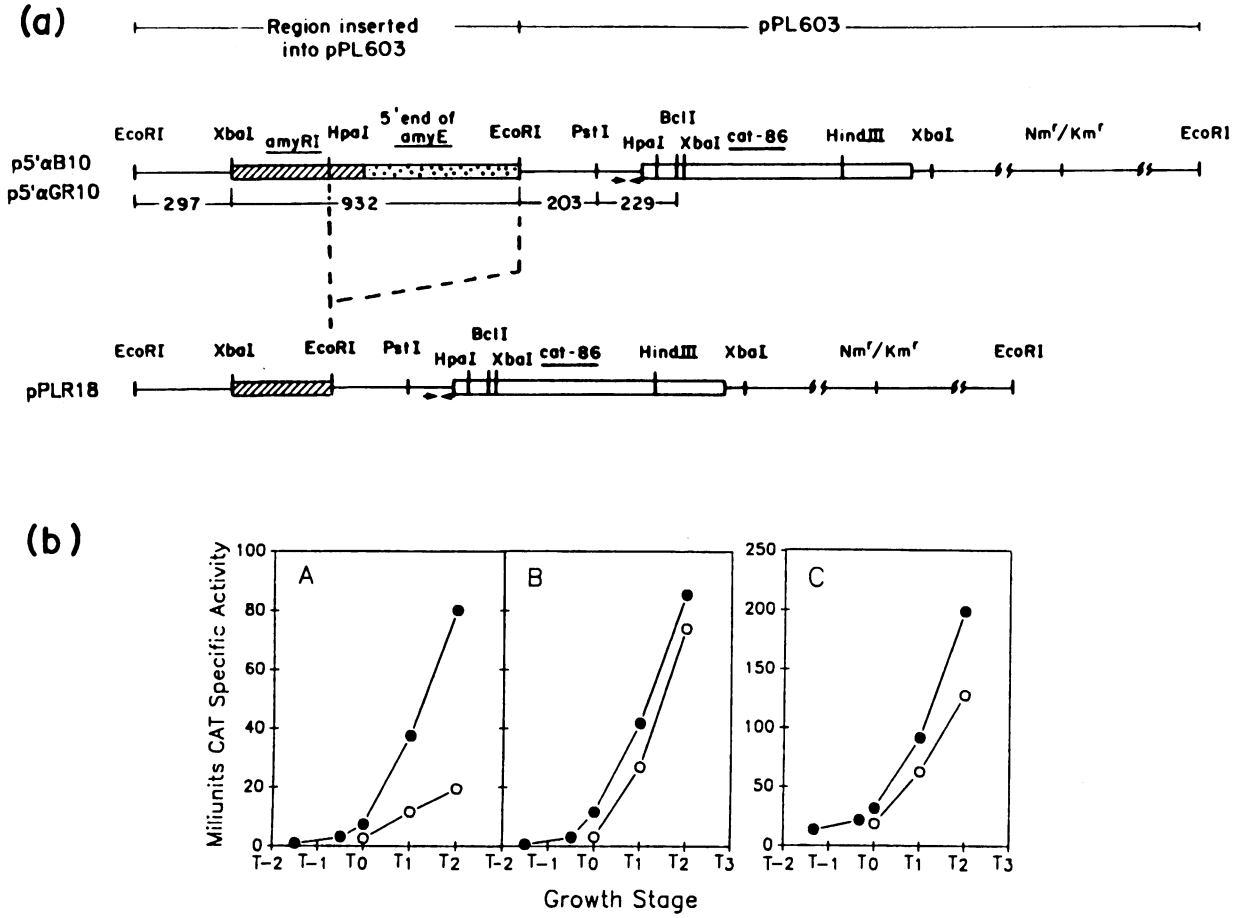

FIG. 7. CAT activity of *amyR–cat-86* transcriptional fusions. (a) Construction of pPLR18 is described in the text. The plasmid, linearized with *Eco*RI, is composed of the 5′ end of the *amyE* gene (stippled bar), *amyR* region (crosshatched bar), *cat-86* gene (open bar), and vector DNA (solid line). The facing arrows are inverted repeats preceding *cat-86*; sizes are specified in base pairs, and Nm^r/Km^r is the neomycin and kanamycin resistance determinant. p5′αB10 and p5′αGR10 are identical fusions of the *cat-86* gene to *amyR1* or *gra-10*, respectively, and were described previously (26, 28). (b) Regulation of CAT synthesis during growth of strain 168 containing (A) plasmid p5′αB10, (B) pPLR18, or (C) p5′αGR10. Open circles are CAT activity in cells grown in NSM with 1% glucose, and solid circles are CAT activity in cells grown in NSM without glucose. Growth stage is expressed as hours before and after T_0, and CAT specific activity was determined as described in the text.

Insertion of 8 bp into the operator region. Since the 3′ *amyR1* deletion may have eliminated catabolite repression because of loss of downstream sequences instead of part of the operator, we inserted an 8-bp *Hin*dIII linker into the same *Hpa*I site. This insertion at the *Hpa*I site, from which the pPLR18 deletion plasmid was made, interrupted the operator 7 bp downstream of the transcription start site (+7), at the same site as the deletion mentioned above, and was confirmed by sequencing. Amylase production in cells of *B. subtilis* BRE containing the plasmid with this 8-bp insert was four- to fivefold higher in logarithmic growth phase than in BRE containing the wild-type *amyR1* plasmid pAMY10 (compare Fig. 8 and 2B). The amount of amylase produced during stationary phase was twice the normal level. Glucose prevented an increase in amylase production after T_0, but the amount produced remained quite high (Fig. 8).

Site-directed mutagenesis. Upstream deletions had no effect on amylase expression, and an insertion at the *Hpa*I site +7 to the transcription start site increased amylase promoter activity. We mutagenized the bases at −7 and +7 in *amyR1* to the corresponding different bases found in *amyR2* to determine whether these changes conferred the characteristic hyperproduction and weaker catabolite repression of the *amyR2* mutation.

The general scheme of oligonucleotide site-directed mutagenesis is shown in Fig. 9. We subcloned *amyR1* and a portion of *amyE* onto pGEM-3Zf(+), a vector which can produce ssDNA or double-stranded DNA in *E. coli*. Mutants were recovered at a high efficiency (50 to 100%) with the Amersham kit. Mutations at +7 were screened by their inability to be digested with *Hpa*I, since such mutations would eliminate the *Hpa*I restriction site (Fig. 6). Mutants determined by screening were confirmed by sequencing. Mutations at −7 were screened by sequencing. Mutations at +7 are called M1; those at −7 are called M2; and double mutants are called M3. Plasmids containing these mutations end with one of these designations (pGEMR1M1, pAR1M2, etc.). The double mutation was constructed by isolating ssDNA from *E. coli* TG1 containing pGEMR1M2 and mutagenizing it with the oligonucleotide carrying the mutant base at +7. Transformant colonies were screened for plasmids that had lost the *Hpa*I site. Suspected mutants were confirmed by sequencing.

In order to examine the effect of these mutations on amylase production, it was necessary to place the *Eco*RI fragment containing *amyR* and part of *amyE* into pARED, a plasmid with this fragment deleted (Fig. 9). Chloramphenicol-resistant, amylase-producing colonies were screened,

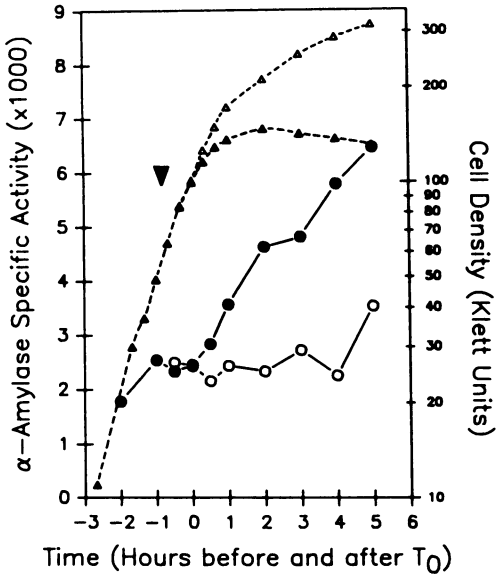

FIG. 8. Regulation of amylase synthesis (circles) during growth (triangles) of *B. subtilis* BRE(pARI7). Symbols and scales are described in the legend to Fig. 2.

and the mutations were reconfirmed by sequencing. Proper constructions, pAR1M1, pAR1M2, and pAR1M3, were transformed into *B. subtilis* BRE.

Amylase production from strains carrying plasmids with site-directed mutations. The amylase specific activity for BRE with plasmids containing single mutations, pAR1M1 (+7 TA⇒GC) or pAR1M2 (−7 TA⇒AT), or the double mutant, pAR1M3 (both of the above mutations), was compared with that from the wild-type pAMY10 (*amyR1*) and pAR2 (*amyR2*) plasmids. All three mutant plasmids directed a level of amylase production well above that of pAMY10 (Fig. 10). The mutation at +7 yielded a level of amylase production in the presence of glucose approaching that by pAR2 (Fig. 10). Although the mutation at −7 doubled amylase production, the amount produced in the presence of glucose was still like that by pAMY10, <10% of the unrepressed level. Amylase production by the plasmid pAR1M3 carrying both mutations was only slightly higher than that by the plasmids with either of the single base changes and was essentially like that of plasmids carrying authentic *amyR2*. The increase in activity for these mutations is apparently not additive.

DISCUSSION

We have recently shown that regulation of *B. subtilis* α-amylase occurs at the level of transcription (29). Transcription occurs in vivo from a promoter 50% homologous to the σ^A (σ^{43})-type RNA polymerase consensus sequence. This promoter is used in vitro by purified Eσ^A (29). It is not yet known why transcription is activated after exponential growth even though vegetative Eσ^A RNA polymerase is most probably used.

In many cases, transcription activation in procaryotes involves a positive control element which binds to a high-affinity binding site upstream of RNA polymerase binding at the promoter. Interaction between the protein factors bound to different sites on the DNA could account for the activation of transcription. Some examples of activation of transcription controlled by factors binding upstream of the promoter include the activation of *ompC* by *ompR* (21), the activation of *nifA* and *glnAp2* (reviewed in reference 9), and many other positively regulated genes reviewed by Ribaud and Schwartz (32).

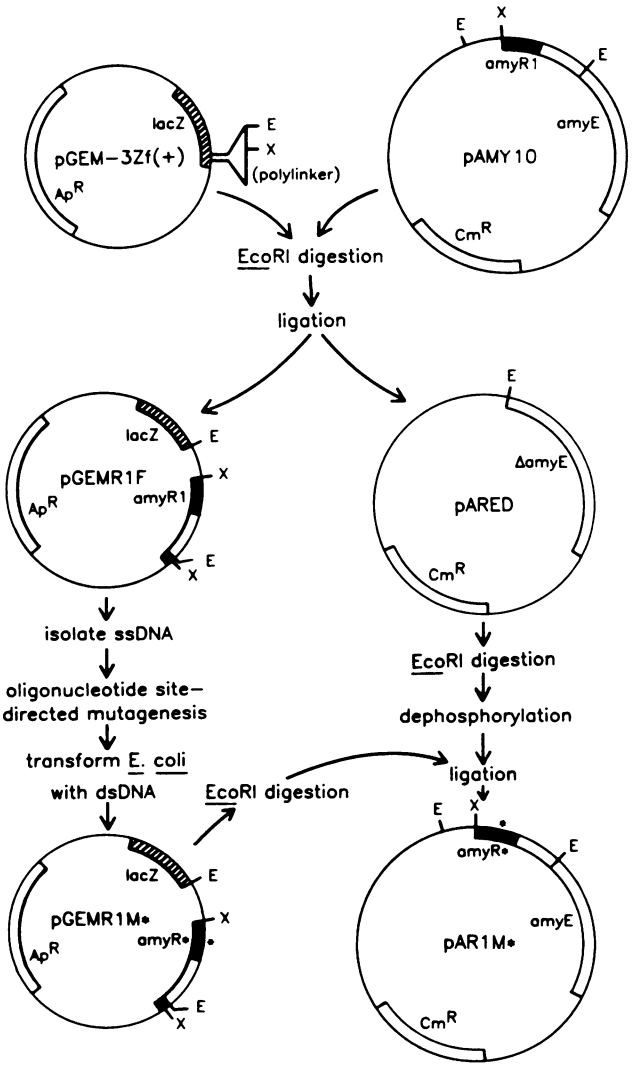

FIG. 9. Construction of plasmids for site-directed mutagenesis and subsequent analysis. Details are discussed in the text. E, *Eco*RI; X, *Xba*I; Ap^r, ampicillin resistance gene; Cm^r, chloramphenicol resistance determinant. The *lacZ* gene is crosshatched, and the *amyR* region is shown as a solid bar. Mutants carrying mutations of the *amyR* region are shown by * and were labeled either 1, 2, or 3 as discussed in Results. dsDNA, Double-stranded DNA.

In *B. subtilis*, A+T-rich regions upstream of promoters have been implicated in transcription activation (3, 24), possibly as a site for protein factor binding. Deletion of the A+T-rich upstream sequences in the *B. subtilis sacB* and *aprE* genes prevents the stimulation of transcription by *hpr-97*, *sacU32*, or *sacQ36* mutations (13). These mutations have been reported to cause hyperproduction of α-amylase (18, 19, 38), and an analysis of the effects of these mutations on amylase transcription is forthcoming (M. J. Weickert, M. W. Song, W. L. Nicholson, and G. H. Chambliss, manuscript in preparation).

It has been speculated that 5′, *cis*-acting sequences, notably the inverted repeat in *amyR2*, are required for regulation.

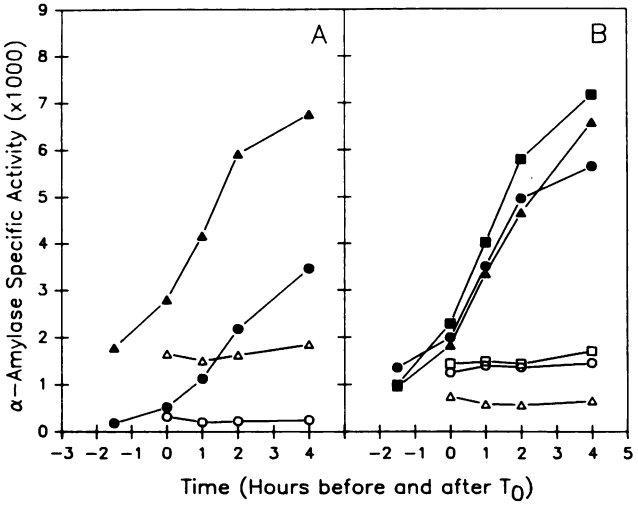

FIG. 10. Regulation of α-amylase for strain BRE containing wild-type or site-directed mutation-carrying plasmids. (A) Amylase from strains containing wild-type *amyR1* (pAMY10; circles) or *amyR2* (pAR2; triangles) with (open symbols) or without (solid symbols) glucose as described in the legend to Fig. 2. (B) Amylase from strains containing *amyR1* mutant plasmids pAR1M1 (circles), pAR1M2 (triangles), and pAR1M3 (squares) grown with and without glucose.

Yamazaki and co-workers (45) hypothesized that the inverted repeat corresponded to the *amyR2* hyperproduction of α-amylase. Later they reported that the presence of the inverted repeat enhanced transcription of the amylase promoter sixfold when starch was present in the medium and lessened the repression in the presence of glucose (39). Under the conditions of our experiments, complete elimination of the upstream inverted repeats for both *amyR1* and *amyR2* does not affect the temporal activation of amylase production, glucose catabolite repression, or hyperproduction in *amyR2* (Fig. 3 and 4). We also determined that the transcription start site and the amount of *amyE* mRNA produced were not affected by the deletions (Fig. 5). Thus, we conclude that the inverted repeats do not contribute significantly to amylase regulation. The enhanced expression seen by Takano et al. (39) in the presence of starch could still be influenced by the inverted repeats. We did not examine the effect of starch on amylase expression in our deletions because we worked with the intact amylase gene and cannot assay amylase accurately from a medium containing starch.

An alternative function for inverted-repeat structures located upstream of promoters is as transcription terminators for upstream operons. In *B. amyloliquefaciens*, the α-amylase gene is preceded by an inverted repeat much like those we studied in *B. subtilis*. This A+T-rich (69%) inverted repeat is located between 78 and 120 bp upstream of the transcription start site (16). This is slightly closer to the amylase transcription start site than that for *amyR1* and *amyR2* and is less A+T rich. The inverted repeat in *amyR1* has 89% A's and T's, and *amyR2* is 83% A+T rich (Fig. 6). In *B. amyloliquefaciens*, the inverted repeat functions as a transcription terminator for a 2.2-kb operon upstream of the α-amylase gene (17). Transcription terminates in a string of T's near the 3′ end of the inverted repeat. There is a series of T's at the 3′ end of the *amyR1* and *amyR2* inverted repeats also (Fig. 6). Therefore, it is plausible that these sequences function as terminators of upstream transcription in *B. subtilis* and have no direct effect on α-amylase transcriptional regulation.

Cells containing the *amyR2* deletion plasmid pAR2110 still activated amylase production at the end of exponential growth, even though the deletion extended through the −35 region of the promoter. Amylase production was also catabolite repressed by glucose in this mutant. The overall level of amylase was severely reduced, probably from the loss of the −35 region, which changes the TTGTTT to ATTTTT (σ^A consensus −35 is TTGACA). Temporal activation and catabolite repression must rely on sequences 3′ to the terminus of this deletion.

Deletion of *amyR1* sequences downstream of +7 did not affect the temporal activation of CAT production in the *amyR1–cat-86* fusion plasmid pPLR18 (Fig. 7bB). This, in conjunction with the 5′ deletion studies above, indicates that sequences required for temporal activation must be located between −27 and +7, the limits of deletion plasmids pAR2110 and pPLR18, respectively. Thus, the promoter itself, a σ^A-type promoter, has some characteristic making it subject to temporal activation, or sequences around the −10 region are important, or both.

The deletion of sequences from the 3′ side of the *amyR* promoter in pPLR18 included the last four bases of the operator region. This deletion eliminated glucose catabolite repression of *amyR1*-directed CAT production (Fig. 7b). The insertion of a *Hin*dIII linker at the *Hpa*I site, from which the deletion was made, reduced catabolite repression of amylase production by glucose and also elevated the level of amylase produced (Fig. 8). The doubling of the level of amylase hinted that the same region required for catabolite repression may be involved in the hyperproduction of amylase in *amyR2* mutants. Two of the single base differences between *amyR1* and *amyR2* are located within the regulatory region defined by the deletions. The amylase operator is also located partially within these limits. Both of these bases were changed in *amyR1* to their *amyR2* counterparts. The first mutation, M1, a TA⇒GC change at +7, is adjacent to the 3′ deletion and insertion endpoints discussed above. This mutation weakened catabolite repression and doubled the amount of amylase produced. This suggested that the hyperproduction in *amyR2* mutants may be related to weakened catabolite repression. The repressor molecule may be present throughout growth and bind weakly to the operator unless it is activated by the presence of easily metabolizable carbon sources, in which case it would bind strongly. This hypothesized weak binding may act to reduce transcription from the *amy* promoter. It should be noted, however, that the *gra-10* mutation does not cause overproduction of amylase (25). Somewhat surprisingly, though, the second base difference, the TA⇒AT change at −7, also doubled amylase production. However, it did not alter the relative level of catabolite repression. This change to an A residue is similar to a TA⇒AT change at −5, which increased *lacUV5*-directed CAT expression in *B. subtilis* 3.5-fold (12). This region may be involved in open complex formation after RNA polymerase is bound to the promoter (41). In gram-positive organisms, 62% of the vegetative promoter −10 regions are followed by an A as the first base (8). An A at this position may facilitate open complex formation in some way. The effect of the two mutations together is not synergistic or even additive; however, together they cause amylase regulation to look most like that in *amyR2* variants (Fig. 10).

The 34-bp region spanning the −10 region of the promoter through the putative operator, and containing the transcription start site, apparently is sufficient to regulate temporal

activation of transcription, catabolite repression, and hyperproduction in *amyR2* mutants. This and previous studies (25) have shown that temporal activation and catabolite repression are genetically separable. Hyperproduction in *amyR2* strains also appears to be independent of temporal activation. It is plausible that all three regulatory phenomena exert their effect by influencing open complex formation.

The regulation of amylase was maintained even when 10 or more copies of the gene were present. α-Amylase regulation presumably involves a regulatory phenomenon shared by other genes; thus, effector molecules involved in amylase regulation might be expected to be present in relative abundance. Activation of expression after the onset of stationary phase is shared by many genes not directly concerned with sporulation. These include enzymes such as succinate dehydrogenase (*sdh* [23]), aconitase (*citB* [33]), proteases, and RNases (reviewed in reference 34). Genes involved in flagellum production and motility (5, 40) and most competence genes (1) are also activated at the onset of stationary phase. Some of these require minor sigma factors, like the *ctc* promoter transcribed after T_0 by $E\sigma^B$ (15, 31), or the competence genes, some of which require $E\sigma^H$ (1).

Catabolite repression by glucose and other readily metabolized carbon sources affects sporulation (20) and a large number of other genes in *B. subtilis*, including *sdh* (23), *gnt* (7), and *citB* (33). Since temporal activation and catabolite repression appear to be global regulatory phenomena, it is not surprising that a 10- to 15-fold increase in the copy number of a single gene does not titrate out the regulatory molecules involved. Whether the same regulatory molecules are involved for all the catabolite-repressed genes is still unknown.

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