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Bacillus licheniformis α-Amylase Gene, *amyL*, Is Subject to Promoter-Independent Catabolite Repression in *Bacillus subtilis*

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Expression of the *Bacillus licheniformis* α-amylase gene, *amyL*, was temporally activated and subject to catabolite repression both in its natural host and when cloned on a 3.55-kilobase fragment in *Bacillus subtilis*. A subclone from which the promoter region of *amyL* and sequences upstream from the promoter were deleted had a low level of amylase activity. Expression of the promoterless gene was still subject to repression by glucose when the gene was present either on a multicopy plasmid or integrated into the *B. subtilis* chromosome. Catabolite repression occurred independently of the amylase promoter and irrespective of the distance of the promoterless *amyL* gene from the promoter which transcribed it. The transcriptional start sites of *amyL* activated by its own promoter and by a vector sequence promoter were determined by S1 mapping. α-Amylase-specific mRNA levels were measured in repressing and nonrepressing media, and catabolite repression was found to act at the level of transcription.

Catabolite repression is the repression of enzyme synthesis that occurs when bacterial cultures are grown in the presence of a readily metabolized carbon source, such as glucose (13). This phenomenon, which was initially described as the glucose effect (6, 19), is a feature of both gram-positive and gram-negative organisms. Early studies on *Escherichia coli* demonstrated that the addition of glucose to bacterial cultures resulted in a rapid loss of intracellular cyclic AMP (cAMP) (14) and that the addition of high levels of cAMP overcame, at least in part, the repressive effects of glucose (35). From these and other studies (reviewed in references 27 and 36), a model emerged in which it was proposed that cAMP binds to a catabolite activator protein (CAP) and that this complex in turn binds to catabolite-sensitive promoters exerting a positive control on catabolic operons. However, to associate catabolite repression solely with cAMP-CAP-dependent regulation of transcription is misleading. Although there is clear evidence that cAMP plays an important role in regulating the transcription of a large number of operons, it has not been established as the sole mediator of catabolite repression. Indeed, mutation studies have demonstrated that neither a functional CAP nor an active adenylate cyclase enzyme is essential to mediate catabolite repression in *E. coli* (4, 9, 38).

Many cellular and developmental processes including sporulation, enzyme transport systems, and extracellular enzyme synthesis are subject to catabolite repression in *Bacillus* species. Unlike *E. coli*, under physiological growth conditions *Bacillus* species do not contain cAMP (1, 10); therefore, cAMP cannot be involved in mediating catabolite repression in *Bacillus* species.

The *Bacillus subtilis amyE* gene is subject to catabolite repression (3, 20). It was shown that when the *Bacillus licheniformis* α-amylase gene, *amyL*, is cloned in *B. subtilis*, its expression is also subject to catabolite repression (S. A. Ortlepp, Ph.D. thesis, University of Dublin, Dublin, Ireland, 1983). Here we confirm these earlier findings and report evidence that catabolite repression of *amyL* occurs at the level of transcription, independently of the promoter from which the gene is transcribed and irrespective of the distance between the structural *amyL* gene and the promoter which activates it.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The following strains were used in this study: *B. licheniformis* FDO2, *B. subtilis* SO113 (*trpC2 amy-3*), *B. subtilis* SO116 [*trpC2 amy-3*(pSA33)] (25), and *B. subtilis* SO122 [*trpC2 amy-3*(pBS86-3)].

Plasmid pSA33 has been described previously (25). pSA33 carries the *B. licheniformis amyL* gene on a 3.55-kilobase (kb) fragment cloned into the *Eco*RI site of pBD64 (8). Plasmid pBS86-3 was a generous gift from M. A. Stephens and was constructed as follows. There is a unique *Nde*I site present immediately 3′ to the −10 promoter region of the *amyL* gene. The 3.55-kb fragment was cut at this site, and a *Bam*HI linker was added (5′-CGGATCCG-3′). A 1.9-kb *Bam*HI-*Hin*dIII fragment was then inserted into the multiple cloning site of pUC8, producing pSL5 (24). The promoterless *amyL* gene was cut out on an *Eco*RI-*Hin*dIII fragment and inserted between the *Eco*RI-*Hin*dIII sites of pBD64 (after conversion of the *Pvu*II site of pBD64 to a *Hin*dIII site), producing pBS86-3 (see Fig. 2a). pOK4 is an integrating vector carrying the promoterless *amyL* gene and has been described previously (24).

Growth conditions. Overnight cultures were grown in Luria broth (LB) plus 5 μg of the appropriate antibiotic per ml. Before inoculation, the cells were pelleted in a bench centrifuge, washed, and suspended in CM medium (Spizizen Salts supplemented with 0.1% sodium citrate, 0.05% casein hydrolysate, and 50 μg of L-tryptophan per ml) or in CM medium plus 1% glucose (1% G). All liquid cultures were vigorously aerated at 30 or 37°C. Growth was monitored spectrophotometrically (optical density at 600 nm [OD_{600}]) or with a Klett-Summerson colorimeter (filtered with a red filter, no. 66). Throughout the growth cycle, 3-ml samples were removed, the cells were pelleted, and the supernatants were stored at −20°C for up to 1 week. The following media

were also used to study growth curves and subsequently α-amylase assays: LB, LB plus 1% G, nutrient sporulation medium (NSM) (31), and NSM plus 1% G. Selective antibiotic media contained 5 μg of chloramphenicol per ml and 50 μg of ampicillin per ml.

α-Amylase assays. (i) Plate test. Cells were plated on LB (LBS) or CM (CMS) agar containing 0.2% soluble starch with or without the addition of 1%G. After overnight incubation at 37°C, the plates were flooded with a 4 mM I_2–160 mM KI solution. Alternatively, the petri dishes were placed over a warm dish containing I_2 pellets. It is possible to detect haloes resulting from starch breakdown by this method without killing the cells.

(ii) Liquid assays. α-Amylase activity was measured by a modification of the method of Smith and Roe (33). Linter starch (0.1%) was prepared by boiling the solution and was kept at >80°C for the duration of the assay. Test tubes containing 1 ml of 0.1 M phosphate buffer (pH 6.9), 0.2 ml of 5 M NaCl, and 1 ml of 0.1% starch solution were preheated to 93°C, and 0.5 ml of appropriately diluted culture supernatant was added. A control blank was treated identically except that 0.5 ml of growth medium was added instead of culture supernatant. After 20 min at 93°C, the test tubes were removed and the reaction was stopped by the addition of 0.01% I_2–0.1% KI in 1 N HCl. Activity (A) per milliliter of supernatant per milligram of starch is defined as:

$$A = \{[OD_{620}\ (\text{blank}) - OD_{620}\ (\text{sample})]/OD_{620}\ (\text{blank})\} \times 2 \times \text{dilution factor}$$

OD_{600} was found to correspond directly with cell number and was used to determine the specific activity (activity per cell) of the enzyme: specific activity = A/OD_{600}.

Glucose assay. To test for the presence of glucose in culture supernatants, 1 ml of supernatant was added to 1 ml of distilled H_2O (dH_2O) and 3 ml of DNS reagent (5 g of dinitrosalicylic acid, 1 g of phenol, 0.25 g of sodium sulfate, 100 g of sodium potassium tartate dissolved in 250 ml of 2% [wt/vol] NaOH, made up to 500 ml with dH_2O, and stored at 4°C) and the tubes were placed in a bath of boiling H_2O for 15 min. The tubes were cooled quickly, and OD_{640} was read. dH_2O (1 ml) was used as a blank, and 1 ml of LB or CM medium was used as a negative control. Known concentrations of glucose were assayed to calibrate a standard curve: glucose concentration (milligrams per milliliter) versus ΔOD_{640} ($\Delta OD_{640} = OD_{640}$ [sample] − OD_{640} [blank]).

Plasmid isolation. *B. subtilis* minipreparations were prepared as described previously (37), and *E. coli* minipreparations were prepared by the method of Birnboim and Doly (2). Large-scale plasmid DNAs were prepared similarly except that the DNA was further purified by CsCl-ethidium bromide density gradient centrifugation (15).

DNA manipulations. Restriction enzymes, T4 DNA ligase, *E. coli* Klenow fragment, polynucleotide kinase, nuclease S1, and polynucleotide linkers were obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.) and used as recommended by the manufacturer. Nuclease S1 was also obtained as a gift from the McCardle Cancer Research Laboratory, University of Wisconsin–Madison. Plasmid DNA was routinely analyzed by electrophoresis in 1% agarose gels cast and electrophoresed in 89 mM Tris–89 mM boric acid–2 mM disodium EDTA and stained in an ethidium bromide (1 mg/ml) bath. DNA fragments for sequencing and subcloning were purified by centrifugation in a sucrose density gradient or by the Gene Clean method (Bio 101, La Jolla, Calif.) after isolation from an agarose gel.

Copy number determination. The plasmid copy number of pSA33 in *B. subtilis* SO113 grown in LB, CM, and CM plus 1% G media was measured as described previously (32).

RNA isolation. RNA was isolated from *B. licheniformis* FD02, *B. subtilis* SO113, *B. subtilis* SO116, and *B. subtilis* SO122 as follows. Each 200-ml culture was grown to the mid-exponential phase (60 Klett units) in NSM, and a 40-ml sample was removed for RNA isolation. The culture was then divided equally, and glucose was added to a 1% final concentration to one half. Samples of 20 ml of cultures growing in NSM and NSM plus 1%G were removed throughout the growth cycle. To each sample, 200 μl of 1 M NaN_3–0.1 M $MgCl_2$ was added, and the culture was rapidly chilled in a dry ice-ethanol bath. After centrifugation, the cells were suspended in 10 ml of protoplasting buffer (15 mM Tris hydrochloride [pH 8], 450 mM sucrose, 8 mM disodium EDTA, 320 μg of lysozyme per ml) and incubated at 0°C for 30 min. After another centrifugation, the protoplasts were suspended in 1 ml of lysis buffer (10 mM Tris hydrochloride [pH 8], 10 mM NaCl, 1 mM sodium citrate, 1.5% sodium dodecyl sulfate) and 30 μl of diethyl pyrocarbonate and incubated at 37°C. After 5 min, the protoplasts were chilled on ice, 0.5 ml of cold saturated NaCl was added, and incubation on ice was continued for a further 15 min. The supernatant was retained after centrifugation at 10,000 rpm (Sorvall SS34 rotor) for 20 min, and 7 volumes of 4 M LiCl were added to precipitate the RNA at 4°C for 15 to 20 h. The RNA was pelleted at 10,000 rpm in a Sorvall SS34 rotor for 90 min, washed in 0.5 ml of 3 M LiCl, and dissolved in 0.4 ml of solubilization buffer (1% sodium dodecyl sulfate, 1 mM disodium EDTA, 10 mM Tris hydrochloride [pH 7.5]). After two phenol extractions and one phenol-chloroform extraction, the RNA was precipitated in 2.5 volumes of 100% ethanol, washed, suspended in 100 μl of sterile dH_2O, and stored at −20 or −70°C. RNA was routinely checked on 1.1% agarose-ethidium bromide (0.5 mg/ml) gels, and the RNA concentration was measured spectrophotometrically (OD_{260} and OD_{280}).

Northern (RNA) blotting. RNA samples were run on vertical denaturing formaldehyde-agarose gels (15) and transferred to a Biodyne nylon membrane (Pall Corp., Glen Cove, N.Y.). Hybridizations were performed as recommended by the manufacturer.

S1 mapping. Cellular RNA (20 to 80 μg), 50 μg of carrier tRNA, and 25 ng of DNA probe (30,000 to 50,000 cpm) were precipitated in 3.4 volumes of 100% ethanol, and the pellet was washed twice with 85% ethanol. The dried pellets were suspended by occasional gentle vortexing in 35 μl of S1 hybridization buffer (80% formamide, 400 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] [pH 6.4], 400 mM NaCl, 1 mM disodium EDTA) and heated at 85°C for 10 min to denature the DNA, and the temperature was allowed to drop slowly to 46°C for overnight hybridization. Nuclease S1 (170 U) was added in 350 μl of S1 digestion buffer (5% glycerol, 0.25 M NaCl, 0.03 M sodium acetate [pH 4.5], 1 mM $ZnSO_4$), and the samples were incubated at 37°C for 30 min. The DNA-RNA hybrids were collected by ethanol precipitation, extracted with phenol-chloroform twice, reprecipitated with ethanol, washed, dried, suspended in 5 μl of loading buffer, and electrophoresed on a 6% polyacrylamide–8.3 M urea sequencing gel.

RESULTS

Temporal activation and catabolite repression of *B. licheniformis* α-amylase gene. *B. licheniformis* FDO2 α-amylase is a

thermostable extracellular enzyme that is not induced by starch. The specific activity of the enzyme was measured in cultures grown in repressing (1%G) and nonrepressing (no glucose) media at different stages throughout the growth cycle. The activity of the enzyme in nonrepressed cultures increased dramatically at the onset of the stationary phase and was subject to repression throughout the growth cycle in cultures growing in medium containing glucose (Fig. 1a).

Temporal activation and catabolite repression of *B. licheniformis* α-amylase gene cloned in *B. subtilis*. Plasmid pSA33 contains a 3.55-kb *B. licheniformis* fragment which includes the *amyL* gene inserted at the *Eco*RI site of the vector pBD64 (8). *B. subtilis* SO116 was made by transforming pSA33 into the α-amylase-negative strain SO113 (25). SO116 was grown in CM medium with and without the addition of 1%G. Culture samples were removed, and the specific amylase activity was measured. The expression of the cloned gene on pSA33 in *B. subtilis* is temporally activated and catabolite repressed in a pattern similar to its expression in its natural host, *B. licheniformis* FDO2 (Fig. 1b). There was a sharp rise in amylase activity as the cells entered the stationary phase in nonrepressing medium. Amylase activity was more than 10-fold higher than in an equivalent culture grown in the presence of glucose. After 32 h of growth in glucose-containing medium, amylase activity remained repressed. When limiting amounts of glucose (<0.1%) were added to the growth medium, amylase activity remained repressed until the glucose present in the medium was exhausted (as determined by the glucose test; see Materials and Methods), and then activity increased to nonrepressed levels. The same results were obtained when LB and LB plus 1%G were used as nonrepressing and repressing growth media, respectively (data not shown).

To test whether this effect might simply be a copy number effect, we measured the copy number of pSA33 in exponentially growing *B. subtilis* SO116 in CM, CM containing 1%G, and LB media (as described in Materials and Methods). There were eight plasmid copies per genome equivalent in all growth media, demonstrating that the low levels of amylase activity in glucose-containing medium are not due to a decrease in plasmid copy number. The 3.55-kb *B. licheniformis* DNA fragment in pSA33 must therefore contain all the *cis* sequences required for catabolite repression of *amyL*. The 5′ end of the gene as well as regulatory and upstream regions of the gene have been sequenced (34). The α-amylase gene is preceded by a 393-base-pair (bp) open reading frame flanked by possible regulatory sequences (Ortlepp, Ph.D. thesis) (Fig. 2b). These sequences are contained within the 3.55-kb fragment.

Catabolite repression of promoterless *B. licheniformis* α-amylase gene in *B. subtilis*. *B. subtilis* SO122 carries a subclone of pSA33, pBS86-3. This plasmid contains the entire α-amylase structural gene and 30 bp upstream from the ATG initiation codon, but not its promoter (Fig. 2a). SO122 is also amylase positive. The expression of *amyL* is presumably activated by an upstream plasmid promoter. Amylase activity from SO122 was measured throughout the growth cycle in cells grown in the presence or absence of glucose. Synthesis of the enzyme was still strongly repressed in the presence of glucose (Fig. 1c). The deletion of the upstream open reading frame sequences did not affect the modulation of *amyL* expression in either a positive or negative manner. Thus, catabolite repression of *amyL* in *B. subtilis* does not require its own promoter or the expression of the upstream open reading frame. The level of amylase activity was much lower (>50-fold) than the level of activity found in SO116, but expression was still subject to catabolite repression.

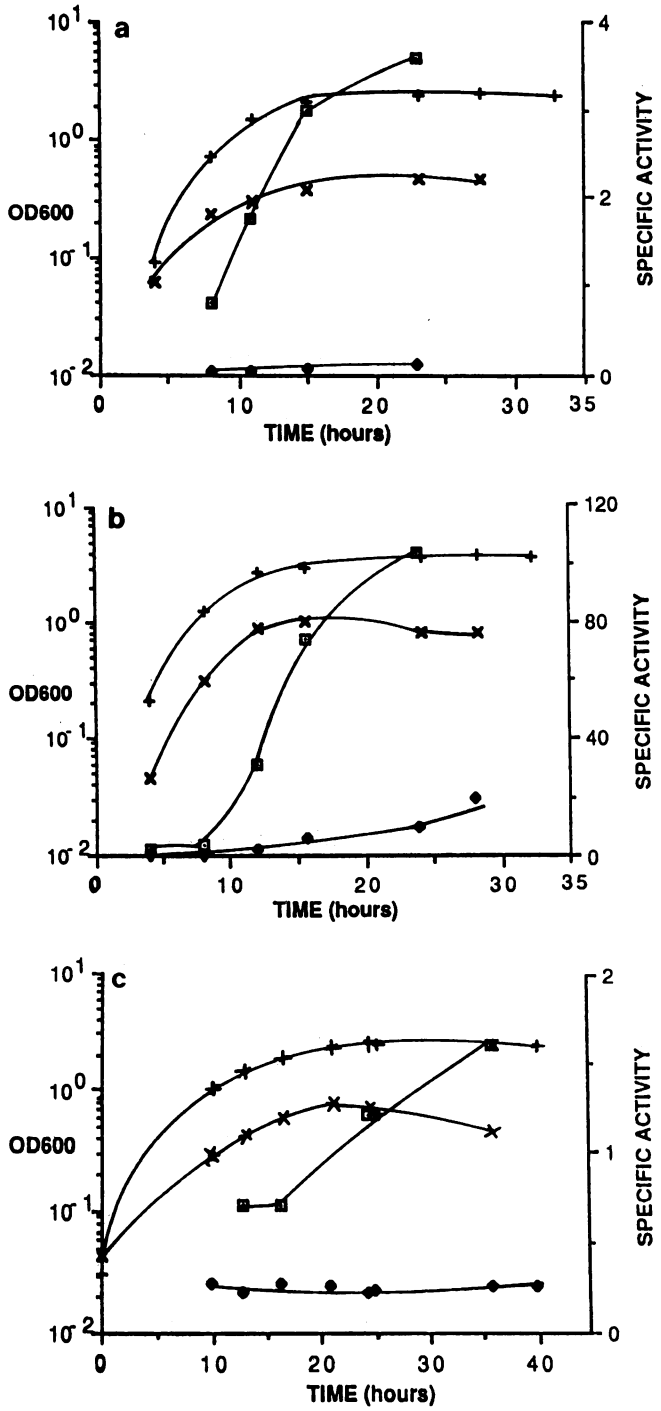

FIG. 1. Growth curves and corresponding α-amylase activity profiles of *B. licheniformis* FD02 (a), *B. subtilis* SO113 (b), and *B. subtilis* SO122 (c). Cultures were grown in CM (×) and CM plus 1%G (+) media, and extracellular α-amylase activity was measured throughout the growth cycle (see Materials and Methods). Symbols: ⊡, specific activity in CM; ◆, specific activity in CM plus 1%G.

O'Kane et al. (24) ligated random *B. subtilis* chromosomal restriction fragments into sites upstream from the promoterless amylase gene in pOK4, transformed SO113, and selected for a plasmid marker (Cm^r). The transformants carried

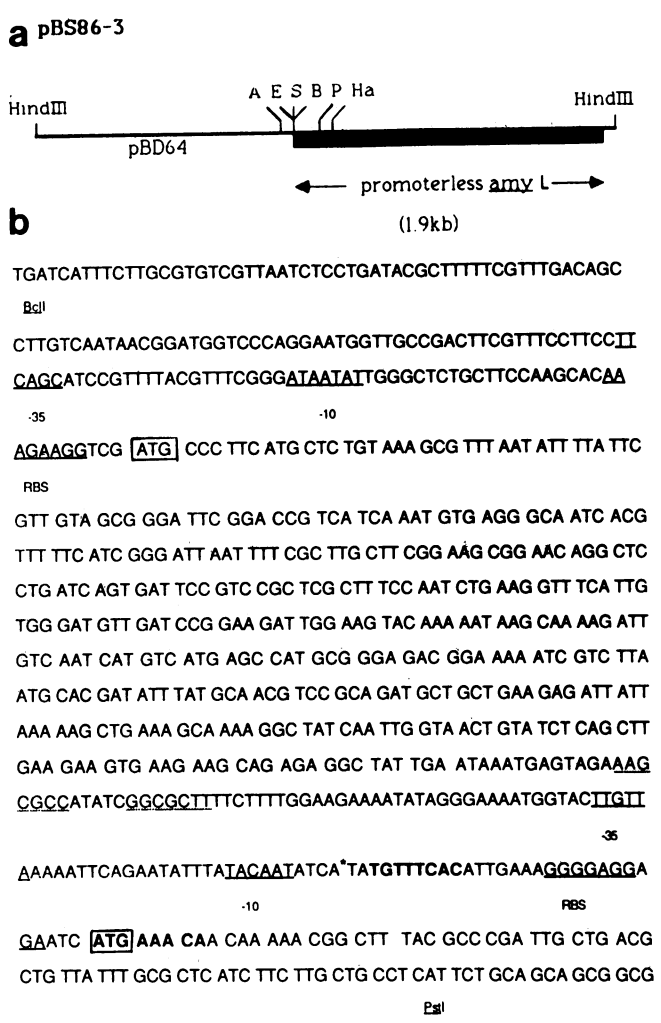

FIG. 2. (a) pBS86-3 (6.7 kb) carries the promoterless *amyL* gene inserted into the *Eco*RI-*Hin*dIII sites of pBD64 (*Pvu*II site converted to *Hin*dIII). A, *Acc*I; B, *Bam*HI; E, *Eco*RI; P, *Pst*I; and S, *Sma*I. (b) Sequence of the 393-bp open reading frame present immediately upstream from the *amyL* gene and the 5′ region of the *amyL* gene. The Eσ^A −10 and −35 consensus sequences and putative ribosome-binding site (RBS) are underlined. A possible terminator IR sequence is shown with dotted underline. Asterisk (*) shows start of the promoterless *amyL* sequence. The IR sequence is shown in bold-face type. The ATG initiation codons are boxed.

the plasmid integrated in a site on the chromosome homologous to the chromosomal fragment carried on the plasmid. A total of 97 randomly chosen amylase-positive transformants were tested on LBS and LBS containing 0.5%G. Qualitative differences in halo size between identical colonies were found for all insertions tested. All the haloes were significantly larger on nonrepressing LB medium compared with those on glucose-containing medium (data not shown). To test this difference quantitatively, we measured amylase activity of stationary-phase cultures of 10 randomly chosen transformants in liquid cultures (Table 1). The activity of the enzyme varied about sevenfold between the transformants, presumably because they were being read from chromosomal promoters of different strengths which are at different distances from the gene. However, it was clear that in all cases glucose exerted a repressive effect on amylase expression (Table 1). This experiment was repeated with a similar

TABLE 1. α-Amylase activities to *B. subtilis* SO113 transformants carrying an integrated *B. licheniformis* promoterless *amyL* gene

Strain no.[b]	α-Amylase sp act (U/mg per ml)[a]			
	CM	CM + 1%G	LB	LB + 1%G
1	1.67	0.28		
2	0.76	0.11		
3	6.55	0.41		
4	6.5	0.5		
5	1.47	0.09		
6			0.75	0.1
7			1.35	0.25
8			0.9	0.3
9			0.55	0.05
10			0.95	0.25

[a] Stationary-phase culture supernatants from strains grown in nonrepressing (CM or LB) media or in repressing (CM + 1%G or LB + 1%G) media were assayed for α-amylase activity.

[b] Randomly chosen α-amylase-positive integrants picked from LBS or CMS plates.

integrating vector (pWD3; kind gift from H. Wood), and the same results were obtained (data not shown).

Transcriptional start site of *amyL*. Plasmids pSA33 and pBS86-3 were linearized with *Pst*I, labeled at the 5′ end (Fig. 3), and used as probes for S1 mapping to determine the in vivo start site of transcription of the *amyL* gene in SO116 and of the promoterless gene in SO122, respectively. Total RNA isolated from these strains and from the parental strain, SO113 (amylase-negative control), was hybridized to the appropriate denatured probe, and nuclease S1-resistant hybrids of approximately 100 bp (SO113, Fig. 4a) and 600 bp (SO112, Fig. 4b) were detected. A single band was protected from each clone. The 100-bp protected fragment corresponds to the expected start site of *amyL* downstream from a σ^A consensus sequence (34). The start site of transcription of the promoterless gene present on the plasmid pBS86-3 is approximately 600 bp upstream from the *Pst*I site. Possible σ^A consensus sequences are present on the vector (pBD64) fragment 568 and 721 bp upstream from the *Pst*I site, and readthrough from one of these promoters is therefore likely to be responsible for the low level of activity found in the promoterless construct. Promoter sequences recognized by other holoenzyme forms were not found from sequence analysis.

Regulation of *amyL* expression occurs at the level of transcription. To determine whether regulation occurs at the level of transcription, we grew *B. subtilis* SO113, SO116, and SO122 and *B. licheniformis* FDO2 in NSM in both the presence and absence of excess glucose. At various times throughout the growth cycle, total RNA was extracted from the bacterial cultures. Equal amounts of total RNA from each sample were hybridized to 3′-end-labeled probes (Fig. 3) to determine the relative amounts of *amyL*-specific mRNA which could protect the probe (present in excess) from S1 digestion (Fig. 5) or when bound to nylon membranes would hybridize to excess labeled DNA (Fig. 6). The results showed that when a 3′-end-labeled probe was used, the levels of S1-resistant mRNA and the amount of hybridization to Northern blots correlated directly with the amylase activity profiles (Fig. 1).

DISCUSSION

We studied the expression of the *B. licheniformis* α-amylase gene, *amyL*, in its natural host and when cloned on

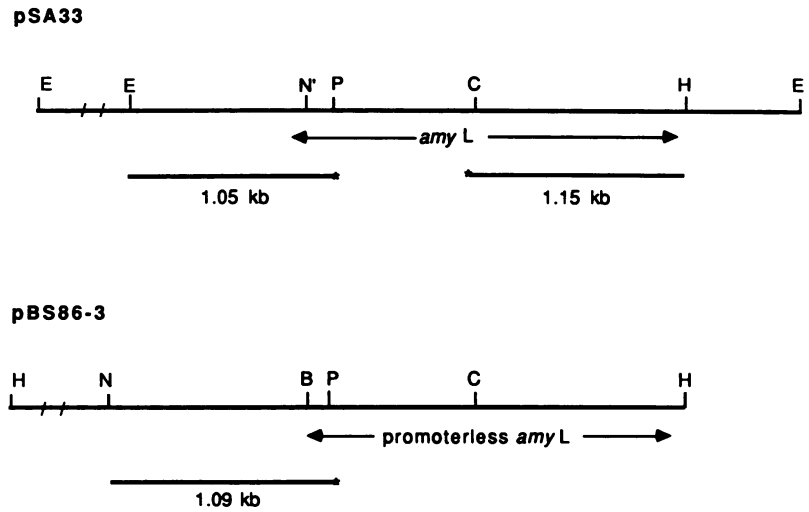

FIG. 3. The 5'-end-labeled and 3'-end-labeled DNA probes used for S1 protection experiments and for Northern blots. The 5' probes were labeled on the antisense strand at the recessed 5' *Pst*I site by using the T4 DNA polymerase exonuclease activity to create a 5' overhang and labeling with [γ-^{32}P]ATP. The 3' probes were labeled on the antisense strand at the *Cla*I site by using *E. coli* Klenow fragment polymerase activity and [α-^{32}P]dCTP to fill in the recessed 3' end. B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; N', *Nde*I; N, *Nsi*I; and P, *Pst*I.

a low-copy-number vector, pSA33, in *B. subtilis*. We utilized this system in an attempt to understand the mechanism of catabolite repression in *Bacillus* species, a phenomenon that is not fully understood in either gram-positive or gram-negative bacteria. The *amyL* gene, present in eight copies per *B. subtilis* chromosome, is subject to both temporal activation and catabolite repression. *amyL* shows very little homology at the DNA level with the corresponding *B. subtilis* gene, *amyRI-amyE* (34), which is also temporally activated and subject to catabolite repression (3, 20). α-Amylase production is repressed 10-fold in glucose-containing cultures in the early stationary phase, similar to the levels of repression of the *B. subtilis amyE* gene (20; unpublished observations). This suggests that the plasmid-borne heterologous gene is not titrating out a regulatory factor present in *B. subtilis* cells.

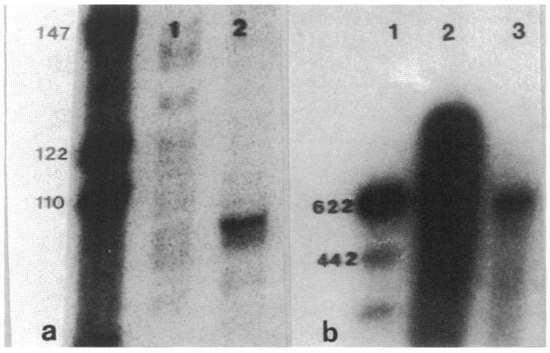

FIG. 4. Results of S1 mapping experiment to determine the start sites of transcription of *amyL* (a) and of the promoterless gene in pBS86-3 (b). (a) A 30-μg sample of total RNA isolated from SO116 was hybridized with a denatured 1.05-kb *Eco*RI-*Pst*I DNA probe end labeled at the *Pst*I site (Fig. 3). After digestion by S1 nuclease, the protected hybrid was run on a 6% polyacrylamide gel (lane 2) along with undigested probe (lane 1) and *Hpa*II-digested pAT153 as size standards. (b) A 50-μg sample of total RNA isolated from SO122 was hybridized with a denatured 1.09-kb *Nsi*I-*Pst*I DNA probe end labeled at the *Pst*I site (Fig. 3). The S1-protected fragment was run out on a 6% polyacrylamide gel (lane 3). Lane 1, Size markers (pAT153/*Hpa*II); lane 2, undigested probe.

A plasmid-borne promoterless construct of the *amyL* gene (pBS86-3) was also subject to catabolite repression. From the size of the S1 transcripts (Fig. 4), it appears that the gene is transcribed by an upstream plasmid promoter which has a consensus σ^A recognition sequence. This suggested that regulation of the gene occurs independently of its own promoter or that the plasmid promoter itself is also subject to temporal activation and catabolite repression. To decide between these two possibilities, we looked at the expression of the promoterless gene when it was randomly integrated into the *B. subtilis* chromosome (24). Amylase-positive transformants, which arise due to transcription of *amyL* by upstream chromosomal promoters, were tested, and all were found to be subject to catabolite repression. Amylase activity between the transformants varied (Table 1), presumably because the gene is being read by promoters of different strengths and/or because the gene is at various distances from these promoters. In many cases, very low amounts of amylase activity were detected, and it was not clear whether the integrated promoterless gene was temporally activated in the transformants. Many *Bacillus* promoters are switched on at specific stages of the growth cycle by particular sigma factors (12, 28), and the activation of these promoters may overide any temporal activation of *amyL* in these integrants. What is clear is that all the integrants are subject to catabolite repression. Glucose-mediated repression of the promoterless α-amylase gene when it is present on a multicopy plasmid or when it is integrated in the *B. subtilis* genome does not require its own promoter and occurs irrespective of the distance between *amyL* and the promoter that is transcribing it. This suggests that the putative regulatory protein involved in mediating catabolite repression binds to a *cis*-acting site present on the promoterless amylase fragment, possibly at or adjacent to the transcriptional start site, resulting in the attenuation of the *amyL* transcript in the absence of a repressing sugar. Alternatively, the regulatory protein contacts the RNA polymerase owing to looping or bending of the DNA (17, 29) so that protein-protein interaction can occur even if the proteins are bound at widely separated sites. In the wild-type gene, the *cis*-acting sequence appears to be adjacent to the promoter (11 [accom-

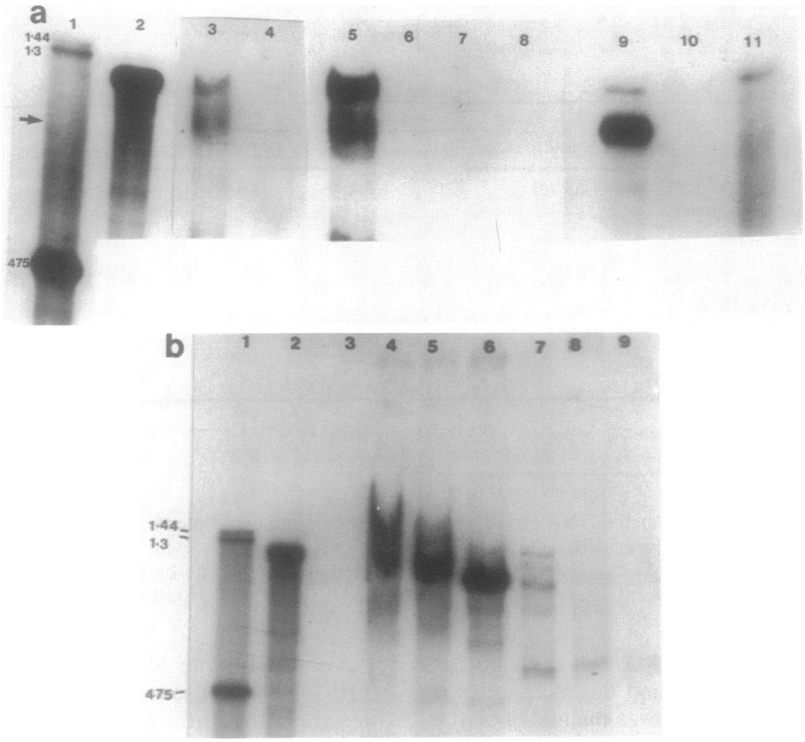

FIG. 5. S1 protection experiments to determine the relative amounts of *amyL*-specific transcripts present in cultures grown in nonrepressing and repressing media. (a) RNA isolated from stationary-phase cultures of SO122 (50 μg; lanes 3 and 4), FD02 (80 μg; lanes 5 and 6), and SO116 (40 μg; lanes 9 and 10) grown in NSM (lanes 3, 5, and 9) and NSM plus 1%G (lanes 4, 6, and 10) (see Materials and Methods) was hybridized with a 1.1-kb *Cla*I-*Hin*dIII-denatured DNA probe labeled at the *Cla*I site (Fig. 3). A 50-μg sample of RNA from SO113 grown in NSM (lane 7) and 50 μg of tRNA (lane 8) were used as negative controls. Lane 2 shows the undigested denatured probe. Lanes 1 and 11 are pBR322/*Taq*I size standards. The arrow shows the position of the protected hybrids. (b) Relative amounts of *amyL*-specific mRNA in SO116 cultures entering the stationary phase (t_0) (lanes 4 and 7), in t_1 cultures (lanes 5 and 8), and in $t_{2.5}$ cultures (lanes 6 and 9) grown in NSM (lanes 4, 5, and 6) and in NSM plus 1%G (lanes 7, 8, and 9). Total cellular RNA (40 μg) was hybridized with the 3′-end-labeled probe in each case. *Taq*I-digested pBR322 was used as a size standard (lane 1). Undigested probe is in lane 2, and lane 3 contains tRNA.

panying report]), and in this case looping out of intervening sequences may not be necessary. A third possibility is that the putative regulatory protein interacts in an indirect manner with the RNA polymerase holoenzyme.

We searched for a possible *cis*-acting site downstream from the promoters of catabolite-repressible *B. subtilis* genes. The data are summarized in Table 2. There is a candidate sequence, 5′-A/TTGTNA/T-3′, in the vicinity of the transcription initiation sites of the genes analyzed. In the case of *amyL*, this sequence is contained within an inverted repeat (IR) sequence and is also present on the promoterless *amyL* fragment. The IR overlaps with the probable start site of transcription of *amyL* and also with the ATG translation initiation codon (Fig. 2b). The IR sequence, 5′-TGTTTCAC-3′, is homologous to a consensus half site (5′-TNTNAN-3′) for DNA-binding proteins that utilize the α-helix-turn-α-helix binding motif (26). An equivalent half-site consensus sequence, 5′-TGTAAG-3′, is present immediately downstream from the P1 promoter of the *B. subtilis amyR1* locus, overlapping the transcriptional start site of the *amyE* gene (22). This motif is also present on an *amyR1* deletion derivative which can still direct glucose-repressible expression of a fused indicator *cat* gene (21). A point mutation (*gra-10*) in this region of dyad symmetry relieves catabolite repression of *amyE* (20, 22).

We determined that the start site of transcription of the *amyL* gene in pSA33 is approximately 110 bp from the *Pst*I site; there is a σ^A consensus sequence immediately upstream

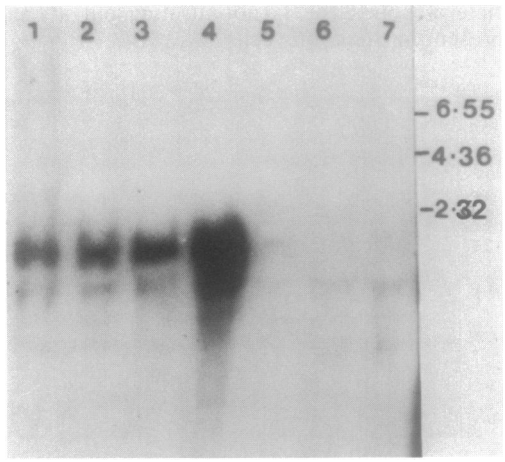

FIG. 6. Northern blot experiment to determine the amount *amyL*-specific transcripts present in SO116 grown in NSM (lanes 1, 2, 3, and 4) and in NSM plus 1%G (lanes 5, 6, and 7). Total cellular RNA (5 μg) from exponentially growing cells (60 Klett units; lane 1), from cells entering the stationary phase (t_0; lanes 2 and 5), from early-stationary-phase cultures (t_1; lanes 3 and 6), and from late-stationary-phase cultures ($t_{2.5}$; lanes 4 and 7) was run out on formaldehyde-agarose gels, blotted onto nylon membranes (see Materials and Methods), and hybridized to a denatured, α-^{32}P-labeled *amyL Cla*I-*Hin*dIII fragment (Fig. 3). Numbers to the right are size standards in kilobases.

TABLE 2. Comparison of sequences downstream from the promoters of *B. subtilis* genes subject to catabolite repression

Gene	Sequence[a]	Reference
amyL	ATGTTT	34
amyR1	ATGTAA	39
citB	ATGTGA	5
gnt	TTGTATA	7
hut	AGTTAATAGTTA	23
sacC	ATGTAC	16
sdh	TTGTCA	18
Consensus sequence	A/T T G T N A/T 7 6 7 7 6 7 7 7 7 7	

[a] The known transcription start sites are shown in bold-face type (for *amyL* both possible sites are indicated).

(34) (Fig. 2b). This agrees with the predicted start site of the *B. licheniformis* 5A1 α-amylase gene (30). Either an A or a G nucleotide in an A+T-rich region immediately downstream from the σ^A −10 region 29 or 31 nucleotides, respectively, from the ATG translation start site is the most likely start point of transcription. The start site of transcription of the *B. subtilis amyE* gene is 121 nucleotides upstream from the ATG codon, which emphasizes the difference between the two genes. We measured α-amylase mRNA levels by S1 mapping and Northern blot experiments with 3′-end-labeled probes (Fig. 3) in strains FDO2, SO113, SO116, and SO122 grown in repressing and nonrepressing media (Fig. 5). The hybridization data showed clearly that in nonrepressed cultures, *amyL*-specific mRNA is transcribed and the levels increase throughout the growth phase in a pattern similar to the observed α-amylase activity profiles (Fig. 6). In the presence of glucose, *amyL* mRNA was barely detectable.

We conclude that regulation of *amyL* occurs at the level of transcription. Catabolite repression of the gene occurs independently of its promoter and irrespective of the distance of the promoterless *amyL* gene from the promoter which activates it.

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