JOURNAL OF BACTERIOLOGY, Nov. 1986, p. 839–842
0021-9193/86/110839-04$02.00/0

Vol. 168, No. 2

Expression of α-Amylase in *Bacillus licheniformis*

DAVID M. ROTHSTEIN,†* PATRICIA E. DEVLIN,‡ AND RICHARD L. CATE
Biogen Research Corp., Cambridge, Massachusetts 02142

Received 8 May 1986/Accepted 13 July 1986

In *Bacillus licheniformis*, α-amylase production varied more than 100-fold depending on the presence or absence of a catabolite-repressing carbon source in the growth medium. α-Amylase was produced during the growth phase and not at the onset of the stationary phase. Induction of α-amylase correlated with synthesis of mRNA initiating at the promoter of the α-amylase gene.

Bacterial α-amylases (α-1,4-glucan-4-glucanhydrolase; EC 3.2.1.1) are secreted proteins that hydrolyze α-1,4 glycosidic linkages, allowing for growth when starch is the sole carbon source. This study involves the expression of the *Bacillus licheniformis* α-amylase, which is of particular interest in industry because of its remarkable thermostability (5, 8). Recently, the α-amylase gene of *B. licheniformis* was cloned, and the promoter region was sequenced (9, 18).

It has generally been reported that expression of α-amylase in various *Bacillus* species, including *B. licheniformis*, is temporally regulated, in that it is expressed predominantly after the exponential growth phase. Previous investigations have also indicated that production of α-amylase in stationary-phase cells is repressed by the presence of glucose, and derepressed when glucose is absent (reviewed in reference 12 and 16).

We show that α-amylase in *B. licheniformis* 5A1 is produced predominantly during growth and not during the stationary phase. Our studies agree with previously published observations that glucose represses α-amylase. Furthermore, we demonstrate that regulation of α-amylase expression is at the level of transcription.

MATERIALS AND METHODS

Strains. *B. licheniformis* 5A1 was obtained from A. L. Sonenshein.

Growth conditions. Cells were grown on 48°C in minimal salts A with buffer A (17) and the following supplements: 10 mM ammonium sulfate; glucose, if present, added to 0.25% unless otherwise indicated; starch, when present, added to 1%; and 0.4% sodium glutamate or sodium citrate. Overnight cultures were grown in medium with glucose as the carbon source.

In experiments in which cells were shifted from glucose to glutamate as the carbon source, overnight cultures contained excess glucose (0.5%) with sodium glutamate added to 0.4%, in minimal medium as described above. One liter of overnight cultures was then added to 9 liters of minimal medium containing 0.1% glucose and 0.4% sodium glutamate. The culture was grown in a Chemap type LF 141 fermenter.

For experiments in which cells were downshifted from ample to limiting glucose, 500 ml of an overnight culture containing 0.5% glucose was added to a total of 9.5 liters of minimal medium containing 0.84 g of glucose per liter. When the culture reached a density of 112 Klett units (green filter, no. 54), approximately 10^8 cells per ml, a slow feed (0.63 g/liter per h) of glucose was commenced.

Assay for α-amylase activity. Samples of cultures were collected and filtered (Millex, 0.4-μm pore size; Millipore Corp., Bedford, Mass.) at the indicated times. The filtrate (up to 100 μl) was added to 0.9 ml of remezol brilliant blue R (13) (20 mg of remezol brilliant blue R [Sigma Chemical Co., St. Louis, Mo.] per ml in 0.02 M $NaPO_4$ [pH 6.9]–0.05 M NaCl) and incubated at 60°C. At various times (up to 20 min), 200 μl of the reaction mixture was removed and quenched by the addition of 1 ml of 0.04 M HCl. After insoluble material was removed by centrifugation, the optical density of the solubilized reagent was determined with a Gilford spectrophotometer (590 nm) and corrected for background.

Activity was standardized for culture turbidity with a Klett-Summerson colorimeter (green filter, no. 54). Units of α-amylase activity are micrograms of remezol brilliant blue R solubilized per minute by 1 ml of culture supernatant per Klett unit (Klett-Summerson colorimeter, green filter, no. 54) of cells ($\mu g \times min^{-1} \times ml$ of $supernatant^{-1} \times$ Klett $units^{-1}$).

Measurement of α-amylase mRNA. Total RNA was isolated by a modification of the method of Palmiter (10). Briefly, *B. licheniformis* was grown to a turbidity of ~100 Klett units as described above, with the exception that the glucose sample contained 0.4% glucose and 50 mM NaCl. Protoplasts were made by incubating cells with 2 mg of lysozyme per ml in SMMP (0.5 M sucrose, 0.02 M maleate, 0.02 M $MgCl_2$ [pH 6.5], and 2× Penassay broth [Difco Laboratories, Detroit, Mich.]) for 10 min at 37°C. After homogenization in lysis buffer (0.2 M Tris hydrochloride [pH 8.0], 0.1 M LiCl, 1% sodium dodecyl sulfate, 25 mM EDTA)-phenol-chloroform (3:1:1, vol/vol/vol), the aqueous phase was reextracted with phenol-chloroform (1:1, vol/vol). Nucleic acid was ethanol precipitated and suspended in 10 mM Tris hydrochloride–1 mM EDTA, pH 7.4.

RNA was analyzed by S1 mapping as described by Brosius et al. (2). The antisense end-labeled probes were prepared from a 415-base-pair (bp) *Sau*3A1-*Pst*I fragment that contains the 5′ end of the α-amylase gene of *B. licheniformis* (see Fig. 3). This fragment was purified from plasmid pPL603-αA (a gift of M. Osburne) which contains the 415-bp *Sau*3A1-*Pst*I fragment inserted between *Bam*HI and *Pst*I sites, permitting its removal with *Bam*HI and *Pst*I. The 415-bp fragment was 5′ end labeled with [γ-^{32}P]ATP and polynucleotide kinase, and the antisense strand (labeled at the *Pst*I site) was purified on a strand separation gel (6). The 415-bp fragment was 3′ end labeled with [α-^{32}P]dATP, [α-^{32}P]dGTP, and DNA polymerase I large fragment, and the

* Corresponding author.
† Present address: Infectious and Neoplastic Disease Research, Lederle Laboratories, Pearl River, NY 10965.
‡ Present address: Cetus Corporation, Emeryville, CA 94608.

TABLE 1. α-Amylase production by *B. licheniformis*

Carbon energy source[a]	α-Amylase activity[b]		Doubling time (min)
	Exponential phase	Stationary phase[c]	
Glucose	11	10	37
Starch	54	52	42
Citrate	143	174	65
Glutamate	525	555	90

[a] Cells were grown at 48°C.
[b] Units are micrograms of reagent solubilized per minute per milliliter of supernatent per Klett unit of culture turbidity (μg × min^{-1} × ml of $supernatant^{-1}$ × $Klett\ units^{-1}$).
[c] At least 1 h after the exponential phase.

antisense strand (labeled at the *Sau*3A1 site) was purified on a strand separation gel (6).

Transcription was measured by S1 nuclease digestion, utilizing probes a and b shown in Fig. 3A. Timed exposures were prepared, and the time required for obtaining equivalent band densities was an inverse measure of transcription. The upstream RNA detected with probe b was used as a standard for total mRNA.

RESULTS

α-Amylase activity with glucose or starch as the carbon source. α-Amylase levels in *B. licheniformis* 5A1 were compared in cultures grown in medium containing either glucose or starch as the sole carbon source. Cells were harvested during exponential growth and in the stationary phase, and the supernatants were tested for α-amylase activity as described in Materials and Methods. Approximately five times as much α-amylase was present per cell mass when cells were grown in medium containing starch instead of glucose

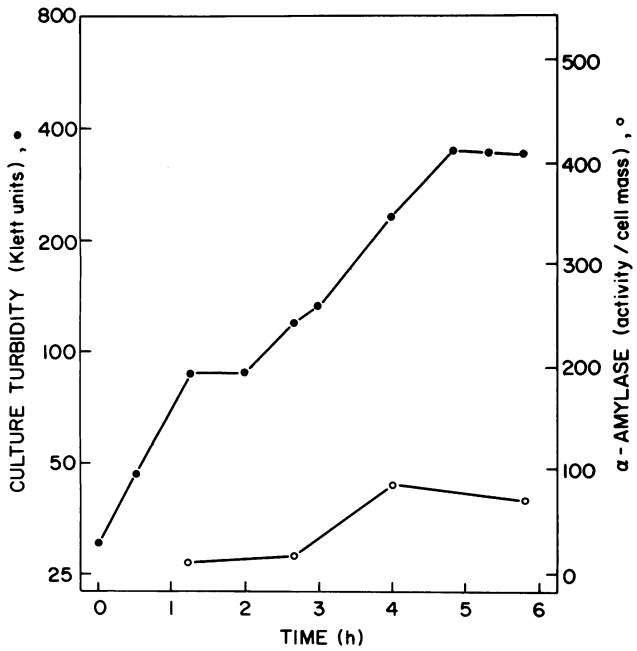

FIG. 1. Growth and α-amylase production of strain 5A1 in medium containing glucose and maltose as carbon sources. Cells were grown in medium containing limiting glucose (0.06%) and 0.4% maltose. Units of α-amylase in culture supernatants are as described in Table 1, footnote *b*.

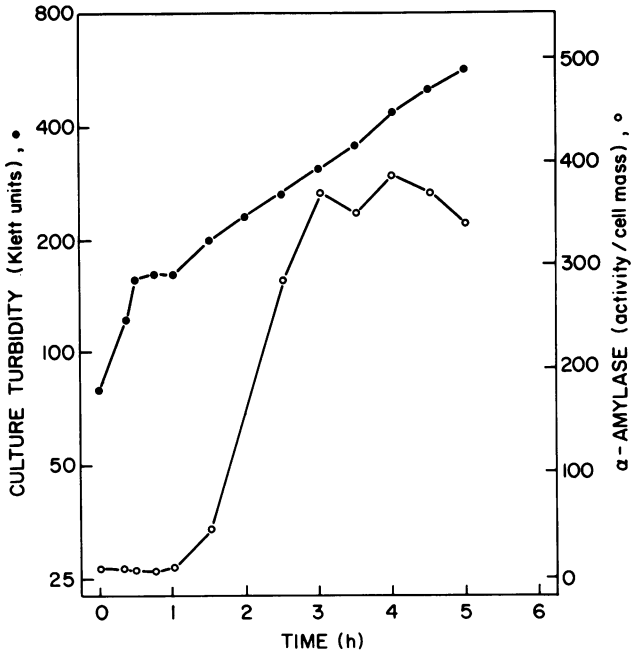

FIG. 2. Growth and α-amylase production of *B. licheniformis* 5A1 in a fermentor with glucose and glutamate as carbon sources. Cells were grown in medium containing limiting glucose (0.1%, enough for partial saturation) and 0.4% sodium glutamate. Units of α-amylase in culture supernatants are as described in Table 1, footnote *b*.

(Table 1). For a given carbon source, α-amylase synthesis remained constant during exponential growth (data not shown). There was no burst of activity at the onset of or during the stationary phase (Table 1).

α-Amylase activity with citrate or glutamate as the carbon source. Induction of α-amylase may be mediated by starch or starch breakdown products. Alternatively, the mechanism of regulation might involve repression of the enzyme, mediated by glucose. In the latter case, the strongest expression of α-amylase might not occur when starch is the carbon source, because starch digestion results in the liberation of glucose polymers which could mediate a degree of catabolite repression.

To test this hypothesis, we measured α-amylase activity in the supernatants of cultures grown in medium containing carbon sources other than sugars. Cells grown in medium containing either citrate or glutamate as the sole carbon source produced more enzyme than cells grown in medium containing starch (Table 1). The more growth limiting the carbon source, the greater the α-amylase production observed. Since the strongest expression occurred when starch was not present in the growth medium, we assumed that starch or starch breakdown products do not induce α-amylase.

α-Amylase activity with glucose and alternative carbon sources in the growth medium. To further test the hypothesis that expression of α-amylase is regulated by glucose catabolite repression, we inoculated cells in medium containing both glucose and maltose. In these experiments, glucose was present at 0.06%. We observed a diauxic growth pattern: rapid growth until glucose was apparently used up, followed by a lag period and a second phase of slower growth on maltose (Fig. 1). α-Amylase was initially produced at the low level characteristic of growth on glucose

during the rapid growth phase and increased to a level per cell mass about eightfold higher during the second growth phase. A parallel experiment in which glucose (0.06%) was added to medium containing starch yielded similar results (data not shown).

A more dramatic diauxic pattern was observed in a 10-liter fermentor when the experiment was repeated with glucose and glutamate. Again, the same pattern of low production of α-amylase during rapid growth on glucose was observed (Fig. 2). Slower growth on glutamate was accompanied by a 150-fold increase in α-amylase production. Thus, when cells were grown on medium containing both glucose and another carbon source, the cells behaved as though glucose was the only carbon source in terms of growth rate and α-amylase production until the supply of glucose was depleted.

To confirm the repression of α-amylase by excess glucose, cells were grown in a fermentor with excess glucose and then shifted to medium containing growth-rate-limiting levels of glucose, as described in Materials and Methods. One hour after the shift, the level of α-amylase increased 60-fold (data not shown).

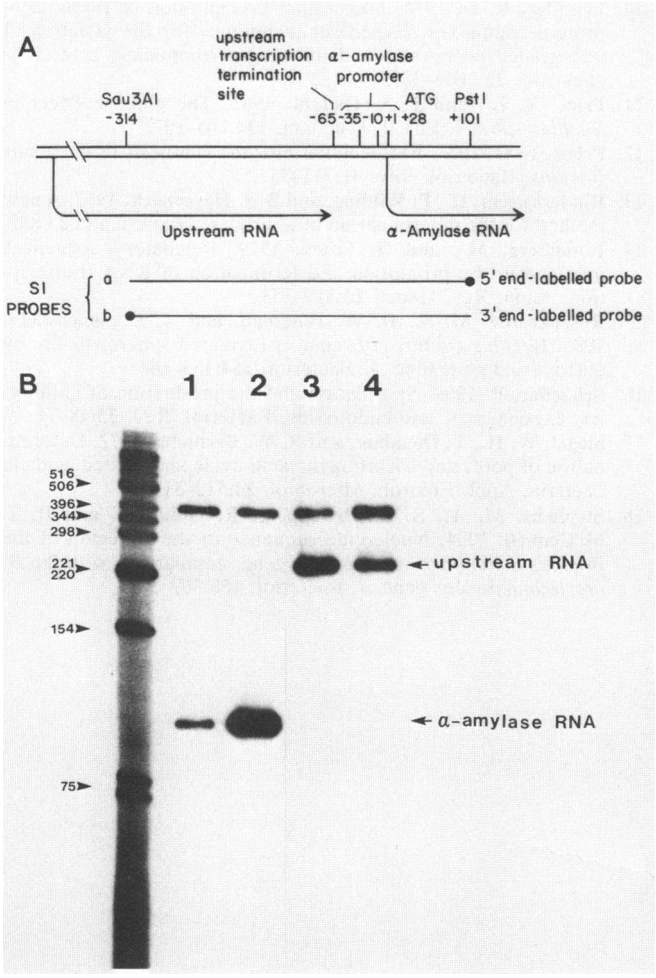

FIG. 3. S1 analysis of RNA extracted from *B. licheniformis* grown in medium containing glucose or glutamate. (A) S1 probes are shown relative to α-amylase and upstream RNAs. The numbering is consistent with the α-amylase RNA initiation site proposed by Stephens et al. (18). (B) S1 nuclease-protected fragments. RNA (20 μg) isolated from *B. licheniformis* 5A1 cells grown in medium containing glucose (lanes 1 and 3) or glutamate (lanes 2 and 4) was hybridized to probe a (lanes 1 and 2) or probe b (lanes 3 and 4) and treated with S1 nuclease. The S1 nuclease-protected fragments were analyzed on a 6% acrylamide–7 M urea gel. The fragments protected by the upstream and α-amylase RNAs are indicated. Numbers on left indicate size markers.

Regulation of α-amylase production is at the level of transcription. To ascertain whether the regulation of α-amylase expression described above occurs at the level of transcription, we performed an S1 analysis. Figure 3 shows a schematic diagram of the α-amylase gene and an upstream transcription unit. RNA extracted from *B. licheniformis* cells grown in medium containing either glucose or glutamate was hybridized to single-stranded end-labeled probe a or b (Fig. 3A) and treated with S1 nuclease. The protected fragments were then resolved on an acrylamide gel (Fig. 3B).

The RNA from cells grown in glucose (lane 1) and glutamate (lane 2) protected a 100-nucleotide fragment of probe a. This places the site for initiation of transcription 100 nucleotides upstream from the *Pst*I site and is in agreement with the position of the promoter suggested by Stephens et al. (18) and Ortlepp et al. (9). Transcription was enhanced at least 50-fold when glutamate was used as the carbon source, consistent with the observed increase in secreted α-amylase described above.

The upstream RNA shown in Fig. 3A served as a positive internal control. It protected a 250-bp fragment of probe b (3′ end labeled), from the *Sau*3A1 site to a transcription termination site located upstream of the α-amylase promoter. S1 analysis showed that there was at least as much of this transcript in cells grown in glucose medium (lane 3) as in the cells grown in glutamate medium (lane 4).

DISCUSSION

We showed that α-amylase production in *B. licheniformis* is related to the nature of the carbon source used for growth. If glucose is present in ample amounts, α-amylase is repressed, even if starch or other carbon sources are also present. When glucose is the carbon source, but present in growth-rate-limiting concentrations, then α-amylase production increases (data not shown). Thus, the concentration of glucose in the medium determines whether α-amylase is repressed. The highest levels of α-amylase were observed when citrate or glutamate was the carbon source. The extent to which catabolite repression is relieved correlates with the degree of growth limitation of the carbon source (Table 1). It is unlikely that starch or other carbon sources such as glutamate play a role in inducing α-amylase. In fact, growth medium containing starch is not a particularly derepressing condition for α-amylase.

Our results differ from the view that α-amylase accumulates during physiological conditions associated with the onset of the stationary phase (12, 16). It is conceivable that *B. licheniformis* 5A1 is regulated differently from most bacilli. However, it is also possible that different growth conditions contribute to the discrepancy between our results and those of others. Strain 5A1 and other *B. licheniformis* strains grow well in defined medium, unlike other bacilli that require undefined supplements that could influence α-amylase production. In addition, starch has often been added to the growth medium in studies of α-amylase, which can complicate the interpretation of the results (Table 1). Our physiological studies agree with those of Meers (7) for another strain of *B. licheniformis* and those of Heineken and O'Connor (3) for *B. amyloliquefaciens*. Both groups showed increased expression of α-amylase owing to limitation of the carbon source in chemostat cultures.

We showed that growth conditions resulting in the appearance of α-amylase in the culture medium result in a corresponding increase in transcription from the α-amylase promoter. Cells utilizing glutamate as a carbon source initiate a considerable number of α-amylase transcripts (Fig. 3), consistent with strong expression of the α-amylase gene. Regulation of α-amylase clearly occurs at the level of transcription. Our data confirm the presence of a transcription termination site upstream of the α-amylase promoter at −65 (Fig. 3), at a location following inverted repeats and a poly(T) tract (9), typical of procaryotic termination sites (14). Thus, the DNA site(s) essential for transcriptional regulation of the α-amylase gene probably resides within the DNA region downstream of the −65 terminator site.

Because α-amylase is not turned on by a specific inducer, it is likely to be regulated at the molecular level by a more general mechanism that controls expression of other catabolite-repressable genes. Examples of such genes are those coding for glutamate dehydrogenase (4), isocitrate dehydrogenase and glucose-6-phosphate dehydrogenase (11), and aconitase (15), all from *B. subtilis*, and protease from *B. licheniformis* (1). It will be interesting to determine the common features shared by the α-amylase gene of *B. licheniformis* and by other *Bacillus* genes regulated by similar physiological parameters.

ACKNOWLEDGMENTS

We are very grateful to Maggie Rosa and Marcia Osburne for supplying plasmids before publication, to Rande Liebowitz and Tom Jack for expert technical assistance, and to Boris Magasanik, Robert Bernlohr, David Levine, and Parrish Galliher for insightful discussions. We also thank M. Osburne for critical reading of the manuscript and N. Ostrom for preparing the manuscript.

LITERATURE CITED

1. **Bernlohr, R. W., and V. Clark.** 1971. Characterization and regulation of protease synthesis and activity in *Bacillus licheniformis*. J. Bacteriol. **105**:276–283.
2. **Brosius, J., R. L. Cate, and A. P. Perlmutter.** 1982. Precise location of two promoters for the β-lactamase gene of pBR322. J. Biol. Chem. **257**:9205–9210.
3. **Heineken, F. G., and R. J. O'Connor.** 1972. Continuous culture studies on the biosynthesis of alkaline protease and α-amylase by *Bacillus subtilis* NRRL B3411. J. Gen. Microbiol. **73**:35–44.
4. **Kane, J. F., J. Wakim, and R. S. Fischer.** 1981. Regulation of glutamate dehydrogenase in *Bacillus subtilis*. J. Bacteriol. **148**:1002–1005.
5. **Madsen, G. P., B. E. Norman, and S. Slott.** 1973. A new heat stable bacterial amylase and its use in high temperature liquefaction. Die Starke **25**:304–308.
6. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
7. **Meers, J. L.** 1972. The regulation of α-amylase production in *Bacillus licheniformis*. J. Microbiol. Serol. **38**:585–590.
8. **Morgan, F. J., and F. G. Priest.** 1981. Characterization of a thermostable α-amylase from *Bacillus licheniformis* NCIB 6346. J. Appl Bacteriol. **50**:107–114.
9. **Ortlepp, S. A., J. F. Ollington, and D. J. McConnell.** 1983. Molecular cloning in *Bacillus subtilis* of a *Bacillus licheniformis* gene encoding a thermostable α-amylase. Gene **23**:267–276.
10. **Palmiter, R. D.** 1974. Magnesium precipitation of ribonucleoprotein complexes. Expedient techniques for the isolation of undegraded polysomes and messenger ribonucleic acid. Biochemistry **13**:3606–3615.
11. **Price, V. L., and J. A. Gallant.** 1983. The glucose effect in *Bacillus subtilis*. Eur. J. Biochem. **134**:105–107.
12. **Priest, F. G.** 1977. Extracellular enzyme synthesis in the genus *Bacillus*. Bacteriol. Rev. **41**:711–753.
13. **Rinderknecht, H., P. Wilding, and B. J. Haverback.** 1967. A new method for the determination of α-amylase. Experientia **23**:805.
14. **Rosenberg, M., and D. Court.** 1979. Regulatory sequences involved in the promotion and termination of RNA transcription. Annu. Rev. Genet. **13**:319–353.
15. **Rosenkrantz, M. S., D. W. Dingman, and A. L. Sonenshein.** 1985. *Bacillus subtilis citB* gene is regulated synergistically by glucose and glutamine. J. Bacteriol. **164**:155–164.
16. **Schaeffer, P.** 1969. Sporulation and the production of antibiotics, exoenzymes, and endotoxins. Bacteriol. Rev. **33**:48–71.
17. **Siegel, W. H., T. Donahue, and R. W. Berhlohr.** 1977. Determination of pools and tricarboxylic acid cycle and related acids in bacteria. Appl. Environ. Microbiol. **34**:512–517.
18. **Stephens, M. A., S. A. Ortlepp, J. R. Ollington, and D. J. McConnell.** 1984. Nucleotide sequence of the 5′ region of the *Bacillus licheniformis* α-amylase gene: comparison with the *B. amyloliquefaciens* gene. J. Bacteriol. **158**:369–372.