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The Regulation of Alpha-Amylase Synthesis in *Bacillus subtilis*

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In *B. subtilis*, α-amylase synthesis is regulated by *amyR* located directly on the upstream of *amyE*. Three different *amyR* alleles have been reported, *amyR1*, *amyR2* and *amyR3*. Strains bearing the *gra-10* mutation which confers derepression for catabolite repression has G→A transition mutation at +5 of *amyR1*. S1 nuclease mapping demonstrated that transcription initiated at 8 bases downstream from the −10 region of putative $E\sigma^A$ promoter P1 in *amyR1* and *gra-10*. In *amyR2*, the major transcription initiatd at the same place and the minor, 10 bases downstream from −10 of P2. The transcript from P2 contributed approximately 15-20% of total *amyE* mRNA. S1 nuclease protection experiment indicated that *amyE* mRNA levels corresponded to the rate of synthesis assumed by specific activities of α-amylase in culture supernatants, suggesting that α-amylase synthesis is regulated at the level of transcription.

Alpha-amylase, an extracellular enzyme capable of hydrolyzing starch to oligosaccharides or maltose, is produced in early stationary phase in *B. subtilis* (12). The *amyR*, which is located on the upstream of *amyE* (α-amylase structural gene), regulates amylase synthesis (19). Three different *amyR* alleles have been reported, *amyR1*, in *B. subtilis*, *amyR2*, in *B. subtilis* subspe. *natto* (15), and *amyR3*, in *B. subtilis* subspe. *amylosacchriticus* (18). The *amyR1* and *amyR2* have been cloned and sequenced (16, 17) and found to be rather homologous. There are, however, sequence differences between *amyR 1* and *amyR2*. In the upstream of promoter region of *amyR2*, there is an A-T rich inverted repeat structure, potentially capable of forming a stem loop structure consisting of 49 bases with free energy of −15.4 Kcal/mol. There is an analogous structure in *amyR1* but it appears to be a subset of that found in *amyR2* and consists of a smaller inverted capable of forming a stem loop structure containing 29 bases with free energy of −7.3 Kcal/mol.

In this study, transcription initiation sites in *amyR1* and *amyR2* were determined. Additionally, because indirect evidence has suggested that temporal activation and catabolite repression are regulated at the level of transcritpion (7-10), a detailed study involving measuring *amyE* mRNA level changes was performed to elucidate the level at which α-amylase synthesis is regulated in *B. subtilis*.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

B. subtilis strains and plasmids used in this study are summerized in Table 1. The maps of plasmids, pMWR1, pMW-10 and pMWR2 were shown in Fig. 1. *B. subtilis* strains were grown in nutrient sporulation medium (13) with vigorous aeration. Cell growth was monitored with Klett-Summerson colorimeter with a No. 66 red filter.

Amylase Assay

The activities of extracellular amylase in culture supernatant were assayed as described previously (8).

Total Cellular RNA Isolation

B. subtilis strain WLN-4, WLN-11 and WLN-15 were grown in NSM either without or with glucose added (1% final concentration) at 60 KU. Cells were harvested at 60 KU, t_0, t_1, and t_2 to measure *amyE* mRNA levels.

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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		
BRE	*trpC2, recE4, lys-3, amyR1-AmyE⁻*	Laboratory stock
WLN-4	*amyR1-AmyE⁺, sacA321*	9
WLN-11	*gra-10-AmyE⁺, sacA321*	8
WLN-15	*amyR2-AmyE⁺, sacA321*	8
Plasmid		
pAMY-10	Cm^R, *amyR1-amyE⁺*	17
pMWR1	Amp^R, *amyR1-AmyE⁺*	9
pMW-10	Amp^R, *gra-10-AmyE⁺*	9
pYKR-2	Amp^R, *amyR2-AmyE⁺*	Laboratory stock

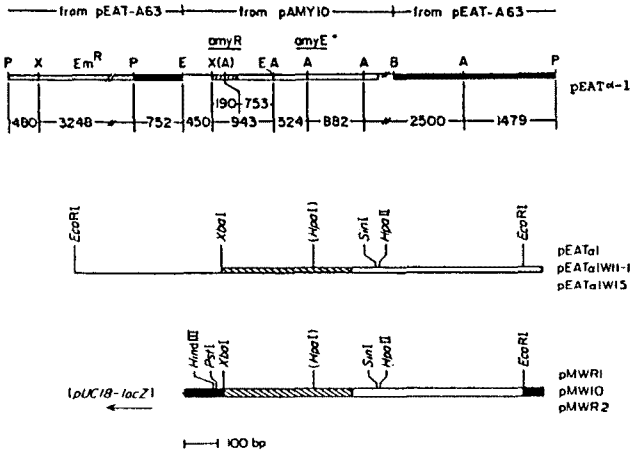

Fig. 1. Restriction map of plasmids used in this study. Plasmid pEATα1 and its derivertives pEATα1W11-1 and pEATα1W15 have been described (ref. 8). Plasmid pMWR 1, pMW-10 and pMWR2 were constructed by insertion of the 933 bp *Xba*I-*Eco*RI fragment from pEATα1, pEATα 1W11-1 and pEATα1W15 respectively, into *Xba*I-*Eco*RI cleaved pUC18.

Total cellular RNA was isolated as described (2, 3).

S1 Nuclease Protection Experiment

The probes for S1 nuclease protection experiments were prepared as described (9). The *PstI-SinI* fragments from pMWR1 and pMW-10, and *XbaI-SinI* fragment from pMWR2, and pMW-10 were labelled by T_4 polynucleotide kinase with [γ-^{32}P]-ATP (3000Ci/mmol, Amersham). S1 nuclease protection was performed as described by Barry *et al.*, (1) with modification. Labelled DNA fragment (10,000-50,000 cpm) and 10-50 μg of total cellular RNA isolated from WLN-4, WLN-11, or WLN-15 were incubated in 35 μl of 80% formaldehyde, 8 mM PIPES, pH 6.4, 50 mM NaCl, 0.2 mM Na_2EDTA at 45℃ for 15-20 hours. S1 nuclease digestion was carried out in 350 μl of buffer containing 7 μl of S1 nuclease (given by Jeff Ross, University of Wisconsin-Madison, U.S.A., S.A. was not determined), 5% glycerol, 0.25 M NaCl, 30 mM NaOAc pH 4.5, and 1 mM $ZnSO_4$ for 30-45 min. Reaction was stopped by adding 1 ml of cold ethanol and analyzed on 7% (w/v) polyacrylamide sequencing gel containing 7 M urea. Sequencing ladders of A+G and C+T were prepared as described (5) with the same labelled DNA fragment used above and electrophoresed for high resolution mapping.

RESULTS AND DISCUSSION

Transcription Initiation Sites

DNA nucleotide sequence analysis reveals three putative promoter sites in *amyR* region potentially recognizable by $E\sigma^A$ RNA polymerase (Fig. 2). S1 nuclease mapping was performed to determine the promoter choice and the *in vivo* transcription initiation sites in *amyR1* and *gra-10* and *amyR2*. When the *PstI* and *SinI* fragment that contains the whole of *amyR* and the N-terminus of amylase was used for S1 mapping, a 201 base fragment was protected from S1 digestion (Fig. 3). Precise size of the protected fragment in a gel was determined by reference to the A+G, and the C+T sequencing ladders of the coding strand. Transcription was initiated at 8 base downstream of putative promoter P1 in both *amyR1* and *gra-10* (Fig. 3A), suggesting that the P1 is the amylase promoter. It was clear that the *gra-10* mutation at +5 (Fig. 2) which confers glucose resistance to α-amylase synthesis did not alter the transcription initiation site. Interestingly, both the P1 and the P2 promoters were apparently used for transcription in *amyR2 in vivo* (Fig. 3B). The primary transcript initiated at the same site as in *amyR1* and *gra-10*. However, a second minor transcript initiated, 10 bases downstream of the P2. The −10 region of P2 in *amyR2* has 5 of 6 homology with consensus sequence, while that of *amyR1* and *gra-10* is 4 of 6 (Fig. 2). This difference, a T in *amyR1* and an A in *amyR2* at position −7, may be sufficient for P2 to be utilized by RNA polymerase *in vivo*. Although P1 diverges more from the consensus sequence for $E\sigma^A$ than does P2, P1 utilization as the primary promoter may be explained by sequences flanking the −35 and −10 regions, especially, the RTRTG sequence located at the one base upstream of −10 (4, 6, 9). The transcript apparently arising from P2 contributes 15-20% of the total *amyE* mRNA pool (Fig. 3B, Fig. 5C).

Total *amyE* mRNA Pools during Growth Cycle

The total *amyE* mRNA levels relating amylase activi-

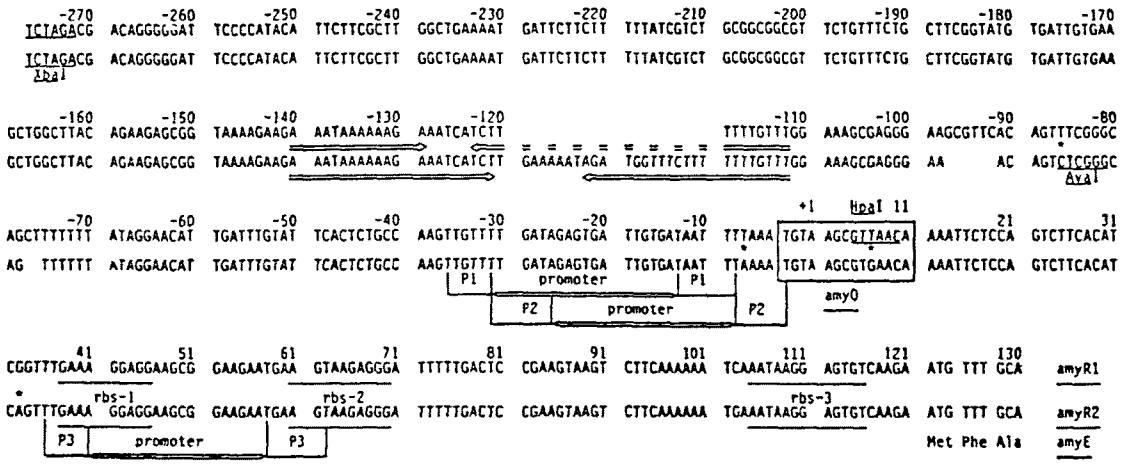

Fig. 2. Nucleotide sequences of *amyR1* and *amyR2*.

Top sequence is *amyR1* and bottom sequence is *amyR2*. Stars (*) indicate base mismatches between *amyR1* and *amyR2*, underlined arrows, the inverted repeat sequences. P1, P2 and P3 represent σ^A type promoter sequences. Transcription initiation sites for *amyR1* and *amyR2* is indicated at +1, for *amyR2*, +1, and +8. The putative ribosome binding sites (rbs−1, rbs−2 and rbs−3) and the recognition sites for restriction endonucleases *Xba*I, *Hin*fI, *Hpa*I and *Ava*I sites are indicated below the sequences. The amino terminal amino acid residues of α-amylase are indicated above the *amyE* sequences.

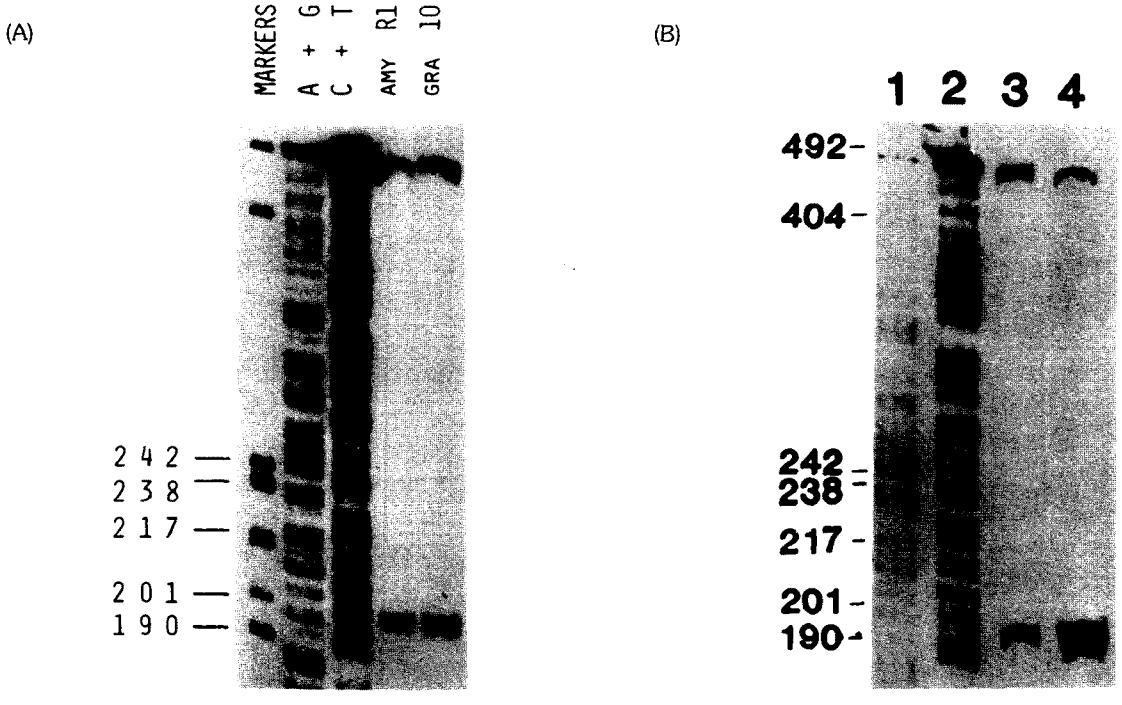

Fig. 3. Determination of transcription initiation sites of *amyR1*, *gra-10* and *amyR2*. As molecular markers pAT153 was digested with *Hpa*II and 3′-ends were labelled.

(A) Transcription initiation sites of *amyRa* (lane 3) and *gra-10* (lane 4) were determined. *Pst*I-*Sin*I fragment was incubated with WLN-4 RNA for *amyR1* mapping, same one from pMW-10, WLN-11. Lane 1 and 2 represent A+G and C+T sequencing ladder respectively.

(B) Transcription sites of *amyR1* (lane 3) and *amyR2* (lane 4) were mapped. *Pst*I-*Sin*I fragment from pMWR2 was incubated with RNA isolated from WLN-15. Lane 1 and 2 represent A+G and C+T sequencing ladder respectively.

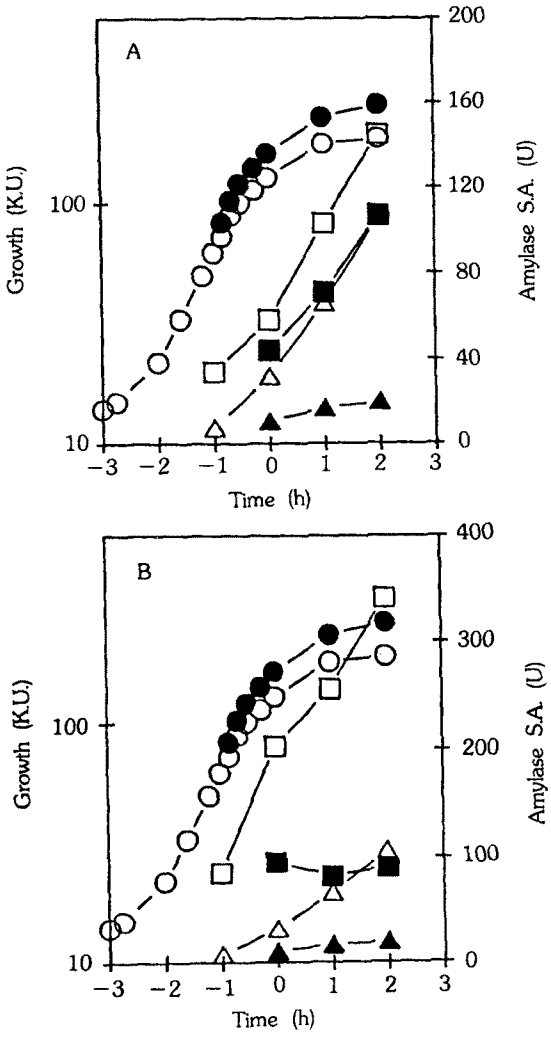

Fig. 4. Amylase production in *B. subtilis* WLN-4(*amyR1-amyE*$^+$), WLN-11(*gra-10-amyE*$^+$) and WLN-15(*amyR 2-amyE*$^+$) in the absence (open) and presence of glucose (closed).

(A) Amylase production in 168 (*amyR1*) and WLN-11 (*gra-10*) Circle, growth; triangle, 168; square, WLN-11

(B) Amylase production in 168 and WLN-15(*amyR2*) Circle, growth; triangle, 168; square, WLN-15.

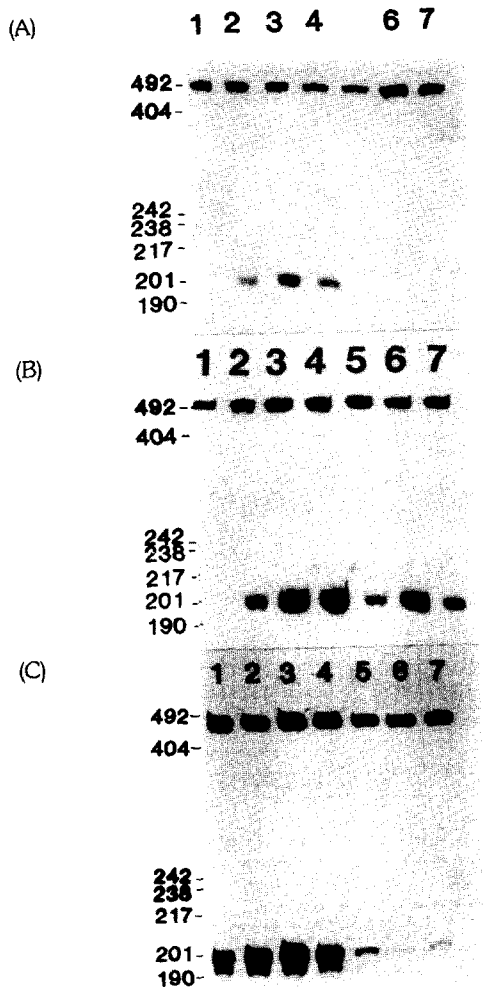

Fig. 5. Measurement of *amyE* mRNA during growth. To quantitate *amyE* mRNA during growth, WLN-4 (*amyR1-amyE*$^+$), WLN-11 (*gra-10-amyE*$^+$) and WLN-15 (*amyR2-amyE*$^+$) were grown with or without glucose and samples were taken to isolate total cellular RNA. Quantitative S1 mapping experiment was performed for each RNA isolated.

(A) *amyE* mRNA of WLN-4. Lane 1, vegetative growth; lane 2-4 cells without glucose, lane 5-7, glucose added. Lanes 2 and 5, t_0; lanes 3 and 6, t_1; lanes 4 and 7, t_2.

(B) *amyE* mRNA level of WLN-11. Lane 1, vegetative growth; lane 2-4 cells without glucose, lane 5-7, glucose added. Lanes 2 and 5, t_0; lanes 3 and 6, t_1; lanes 4 and 7, t_2.

(C) *amyE* mRNA level of WLN-15. Lane 1, vegetative growth, lane 2-4 cells without glucose; lane 5-7, glucose added. Lanes 2 and 5, t_0; lanes 3 and 6, t_1; lanes 4 and 7, t_2.

ties (Fig. 4) during growth were determined by S1 nuclease protection experiment (Fig. 5). In WLW-4 (*amyR1-amyE*), mRNA level increased rapidly at t_0, and was repressed below detectable levels in the presence of glucose (Fig. 5A). But, in WLN-11 (*gra-10-amyE*$^+$), the *amyE* mRNA level was relatively unrepressed by glucose, resulting in 70% of total transcript detected in the presence of glucose (Fig. 5B). Strain WLN-15 (*amyR2-amyE*$^+$), which hyperproduces α-amylase 3 times, had higher *amyE* mRNA levels than WLN-4 or WLN-11 and was slightly less sensitive to glucose repression resulting in 10-15% as much as *amyE* mRNA being synthesized in the presence of glucose as in the absence (Fig. 5C). The rate of amylase synthesis correlated with the slope where specific activity vs time was plotted during the growth cycle (Fig. 4) corresponded to *in vivo amyE* mRNA levels determined by quantitative S1 nuclease

protection experiment (Fig. 5A, B, C).

The increase of accumulated *amyE* mRNA in the early stationary phase could be explained by an increased transcription rate of *amyE* mRNA or by increased stability of *amyE* mRNA. The half-life of *amyE* mRNA in cells growing exponentially is about 24 min. while that of cells during early stationary phase is 12 min. (unpublished results). The possibility of increased stability can be eliminated as the basis for the increased levels of *amyE* mRNA in the early stationary phase. It was also demonstrated that glucose had no direct effect on *amyE* mRNA turnover. Therefore, we conclude that α-amylase synthesis in *B. subtilis* is regulated primarily at the level of transcription.

It is not known why transcription is activated after exponential growth even though the primary *amyE* promoter is recognizable by $E\sigma^A$ RNA polymerase which is predominant in vegetative cells. The are possible models. First, transcriptional activation is carried out via a positive regulatory protein which is produced after exponential growth. The activator protein could bind the A-T rich inverted repeat structure of upstream of the promoter to activate transcription by interacting with RNA polymerase as demonstrated in the other systems (11). Since the deletion of the A-T rich inverted repeat structure and upstream of promoter in *amyR1* and *amyR2* had no effect on temporal activation (14), this model does not seem to fit the regulation of α-amylase. But, we can not rule out the involvement of an activator protein which could bind to another place. Alternatively, transcription of *amyE* may be blocked by a negative regulatory protein bound near the promoter during exponential growth. During the early stationary phase, this protein is detached from the promoter resulting in the activation of transcription. We currently have no data either supporting or refusing this model.

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