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Genetic Control of the Rate of α-Amylase Synthesis in *Bacillus subtilis*

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The level of extracellular α-amylase (EC 3.2.1.1) of *Bacillus subtilis* Marburg was increased about fivefold by introducing the *amyR* marker from *B. natto* 1212 through transformation. *amyR2* of *B. natto* 1212 has been assumed to determine a high level of α-amylase of the organism. The gene acts specifically on α-amylase synthesis but not on the production of other extracellular enzymes. α-Amylase of an *amyR2*-carrying strain was found to be quite similar to that of an isogenic *amyR1*-carrying strain in the thermostability and electrophoretic behavior of whichever amylase the strain produces, Marburg-type α-amylase (*amyEm*) or *B. natto*-α-amylase (*amyEn*). Anti-amylase serum titration indicates that a high level of the enzyme activity in the *amyR2*-carrying strain is caused by the existence of more enzyme rather than the presence of an enzyme having higher efficiency. This is supported further by the fact that *amyR* controls the synthesis of the *amyE* gene product in mutant M9, which synthesizes a temperature-sensitive-α-amylase, and in mutant M07, which secretes cross-reacting material. The results indicate that *amyR* regulates the rate of α-amylase synthesis.

Certain species of the genus *Bacillus* produce various extracellular enzymes such as α-amylase (EC 3.2.1.1), protease, and ribonuclease. The levels of these enzyme activities are known to vary greatly from one strain to another, but little information is available on the genetic control of extracellular enzyme synthesis.

Previously (16), it was shown that *B. natto* 1212 produced about five times as much α-amylase as did *B. subtilis* Marburg. Both α-amylases produced by these two strains were the so-called bacterial saccharifying-type of α-amylase (4, 5); however, the two enzymes can easily be distinguished by their thermostability and electrophoretic mobility. A character that determines the high level of enzyme activity could be transferred from *B. natto* 1212 to *B. subtilis* through deoxyribonucleic acid (DNA)-mediated transformation. About one-tenth of the transformants with this character produced only the recipient-type enzyme, i.e., Marburg (M)-type α-amylase, and the others produced the donor type enzyme, i.e., *B. natto* (N)-type α-amylase. Further genetic analyses indicated that (i) the structural genes (*amyEm* and *amyEn*) for M-type and N-type α-amylase are allelic to each other, and (ii) a gene regulating the level of α-amylase is closely linked to the structural gene. The regulator gene was designated as *amyH* by Yuki (20) or *amyR* by the authors (16), where *amyR1* of *B. subtilis* Marburg and *amyR2* of *B. natto* 1212 direct a low and high level of enzyme activity, respectively. However, because of a close linkage of *amyR* to *amyE*, we assumed that *amyR* occupies a part of the structural gene and determines the specific activity of the enzyme by changing a part of the enzyme protein structure.

In this study, α-amylase produced by isogenic strains bearing *amyR1* or *amyR2* was titrated by anti-amylase serum to compare the specific activities of the α-amylases. Effects of *amyR* on the synthesis of altered α-amylases caused by mutation in the structural gene *amyE* were also investigated. The results indicate that *amyR* regulates the rate of synthesis of the *amyE* gene product.

MATERIALS AND METHODS

Bacterial strains. Strains of *B. subtilis* used in this study were derivatives of strain 168 of Burkholder and Giles (2). Strains 6160 (*metB5 purB6 trp-160 amyEm*$^+$ *amyR1*) and 1-131 (*met his aro-116 amyEm*$^+$ *amyR1*) were donated from Y. Ikeda and S. Yuki, respectively. Strain NA64 (*metB5 purB6*

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amyEm$^+$ *amyR2*) is an *amyR2* derivative of strain 6160, and strain NA6408 (*metB5 aro-116 amyEm*$^+$ *amyR2*) is a derivative of strain NA64. *B. natto* IAM 1212 (*amyEn*$^+$ *amyR2*) is a stock culture at the Institute of Applied Microbiology, University of Tokyo (1). Mutants M9 and M07 were isolated from strains NA64 and NA6408, respectively, by treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (16).

Culture conditions and preparation of α-amylase. Bouillon-yeast extract (BY) medium (7) and glutamate-citrate medium (11) were used for liquid cultures. Bacterial cells were grown overnight with aeration in BY medium at 30 C, and the culture was diluted 100-fold with fresh BY or glutamate-citrate medium. The organisms were cultivated for 24 to 30 h at 30 C, when the maximal activity of the enzyme was reached. The culture was chilled in an ice bath and centrifuged at 6,000 × *g* for 10 min to remove the cells. The supernatant fluid was used as a crude enzyme solution. When necessary, the solution was brought to 90% saturation with ammonium sulfate, and the precipitate was dissolved in 0.04 M phosphate buffer (pH 6.0). It was used after extensive dialysis against the same buffer at 4 C.

Assay of enzyme activity. Determination of α-amylase activity was described previously (16). Hydrolysis of 100 μg of soluble starch in 1 min at 40 C was defined as 1 U of enzyme activity. Since the differential rate of α-amylase synthesis in *B. subtilis* and in *B. natto* increased rapidly at the early stationary phase of growth, we expressed α-amylase productivity in two different ways: (i) the rate of enzyme synthesis (units per milliliter per hour) at the early stationary phase, and (ii) the specific enzyme activity (units per milligram of dry cells) at a time when the enzyme activity reached a maximal level. Protease activity was assayed by a modified method of Hagihara (6).

Procedure of transformation. Transforming DNA was extracted from donor strains with phenol saturated with 0.1 M tris(hydroxymethyl)aminomethane buffer (pH 9.0) containing 1% sodium lauryl sulfate as described by Saito and Miura (13). Transformation experiments were performed by the method of Saito et al. (12). Transformed cultures were plated on a minimal salts and glucose medium (14) supplemented with all growth requirements.

Isolation of strain M9-19. Strain 1-131 was transformed by the DNA from strain M9, and the *aro*$^+$ transformants (600 colonies) were selected (*aro-116* is a marker linked to both *amyE* and *amyR*). They were restreaked on two BY agar plates containing 0.2% soluble starch. After one plate was incubated at 30 C for 24 h and the other was incubated at 45 C for 12 h, the colonies were tested for productivity of α-amylase after being sprayed with 0.01 M I_2-KI solution. Unstained halos were detected around colonies of strain 1-131 grown either at 30 or 45 C, similar unstained halos were observed around colonies of strain M9 grown at 30 C, but only small red halos were observed at 45 C. A small number of organisms producing smaller amounts of α-amylase (3.0% of *aro*$^+$) were detected among *aro*$^+$ transformants in addition to colonies showing the same phenotype as the parents. Small red halos were detected around their colonies grown at 30 C, but none was detected at 45 C. A representative strain, M9-19, was purified. The presence of *amyR1* on the chromosome of strain M9-19 was indicated by the fact that its DNA preparation could transform strain NA6408 (*amyR2*) to *amyR1*.

Polyacrylamide gel electrophoresis. The procedure for polyacrylamide gel electrophoresis was described previously (16). The crude enzyme preparations were subjected to a current of 2 mA per column in 25 mM tris(hydroxymethyl)aminomethane-glycine buffer (pH 8.3) for 13 h at 4 C. After the gel was cut into slices of 5-mm width and incubated overnight in 2 ml of 0.04 M phosphate buffer (pH 6.0) at 4 C, the enzyme activity of each fraction was assayed. Recovery of enzyme activity was 40 to 80% of the input.

Preparation of anti-amylase serum. A mixture of α-amylase (9.6 mg in 0.5 ml of 0.85% NaCl) and 0.5 ml of complete Freund adjuvant (Iapron Laboratory) was injected subcutaneously into a rabbit. The α-amylase was the so-called bacterial-saccharifying type (4) produced by *B. subtilis* var. *amylosaccharitícus* and was purchased from Seikagaku Kogyo Co. Two weeks later, the rabbit received an additional subcutaneous injection of the α-amylase-adjuvant mixture (5.9 mg of α-amylase). Blood was collected 2 weeks after the second injection. The serum was separated, incubated at 56 C for 30 min to inactivate complement, and stored at −20 C. The serum contained a negligible amount of amylase activity compared to α-amylase used in our assay system. Antiserum was usually diluted with 0.85% NaCl solution.

Neutralization of α-amylase with anti-amylase serum. A 0.5-ml volume of the α-amylase solution was mixed with the same volume of the antiserum. After incubation at 40 C for 30 min, the remaining enzyme activity was assayed. The neutralization was almost complete within 30 min under these conditions. Even when excess antiserum was present, the extent of neutralization of α-amylase in the mixture was about 90%; however, after the reaction mixture was centrifuged at 6,000 × *g* for 15 min, no α-amylase activity was detected in a supernatant fluid. Thus, the centrifugation step was included in the experiments presented in this paper.

Estimation of CRM content. A known amount of α-amylase was mixed with various amounts of a culture fluid of a given strain, and the anti-amylase serum was added. The reaction mixture was incubated at 40 C for 30 min, and the α-amylase activity remaining was measured. Since the cross-reacting material (CRM) is assumed to inhibit neutralization of the indicator α-amylase, 1 U of CRM was defined as the amount of material that was antigenically equivalent to 1 U of α-amylase.

RESULTS

Immunological comparison of α-amylases produced by amyR1 and amyR2 strains. As reported previously (16), the *amyR2* marker directing the high level of α-amylase could be

transferred to *B. subtilis* by DNA from *B. natto* 1212 together with the structural gene of α-amylase at a high frequency. Furthermore, *B. natto* produced N-type α-amylase which was readily distinguishable from M-type α-amylase synthesized by *B. subtilis* Marburg. Thus, we could construct four kinds of strains by DNA-mediated transformation. The four strains produced different levels as well as different types of α-amylase. The α-amylases synthesized by them were compared. The strains used in this experiment were as follows: strain 6160 (*amyEm*$^+$ *amyR1*) and its *amyR2* transformants, NA209 (*amyEm*$^+$ *amyR2*) and NA20 (*amyEn*$^+$ *amyR2*), and strain NA2009 (*amyEn*$^+$ *amyR1*), an *amyR1* transformant derived from strain NA20. Table 1 clearly shows that strains NA209 and NA20 are high producers of α-amylase like *B. natto* 1212, whereas strains 6160 and NA2009 are low producers. The genotype *amyE* was deduced from the thermostability and electrophoretic mobility of the enzymes produced. α-Amylase of strain NA209 was similar to that of strain 6160, i.e., M-type α-amylase, whereas α-amylase of strains NA20 and NA2009 were similar to that of *B. natto*, i.e., N-type α-amylase (Table 1, Fig. 1). A heat-labile character of the latter was not due to the presence of a factor in the crude enzyme preparation, since a biphasic inactivation curve was observed when a 1:1 mixture of α-amylase preparations from strains 6160 and NA20 or NA209 was incubated at 55 C (data not shown). Consequently, we could deduce the genotype of these strains with respect to amount and type of α-amylase as presented in Table 1.

Next, immunological studies on α-amylases were carried out to compare their specific enzyme activities. Despite some differences between M- and N-type α-amylases as mentioned above, both types of α-amylase are known to cross-react with antiserum for a saccharifying α-amylase (4, 5) produced by *B. subtilis* var. *amylosaccharitícus*, but they do not cross-react with antiserum for a liquefying α-amylase produced by *B. amyloliquefaciens* (H. Matsuzaki et al., unpublished observation).

Figure 2 shows neutralization curves of α-amylases with antiserum for the saccharifying α-amylase. No significant difference was detected between the neutralization curves of α-amylases whether the enzyme producer bore *amyR1* or *amyR2*. This was true whether the type of α-amylase was M or N, although the neutralization curves of the two types of α-amylases were a little different. Upon preincubation with α-amylase (NA209), the antiserum could not neutralize any α-amylase (6160) added afterwards to the reaction mixture, and vice versa (Table 2). Therefore, these two enzymes are antigenically similar. These results indicate that *amyR* had no effect on the characteristics of α-amylase protein involving its specific enzyme activity.

Effect of amyR on the synthesis of an abnormal α-amylase. A mutant M9 (17) obtained from strain NA64 (*amyR2*) by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment synthe-

TABLE 1. *α-Amylase in amyR1 and amyR2 strains*

Strains	Genotype	α-Amylase productivity		Thermostability[a] half-life at 55 C (min)
		U/ml/h[b]	U/mg of cells[c]	
B. natto 1212	*amyEn*$^+$*amyR2*	4.58 (3.32)[d]	49.1	2.8 ± 0.2
B. subtilis 6160	*amyEm*$^+$*amyR1*	0.57	10.0	32 ± 3
NA209[e]	*amyEm*$^+$*amyR2*	3.59	47.0	40 ± 5
NA20[e]	*amyEn*$^+$*amyR2*	3.36	43.5	2.7 ± 0.3
NA2009[f]	*amyEn*$^+$*amyR1*	0.55	9.80	2.9 ± 0.2
M9	*amyE9 amyR2*	0.66	7.53	2.5 ± 0.1
M9-19	*amyE9 amyR1*	0.14	1.25	2.9 ± 0.2

[a] The enzyme solution (6 to 9 U) was incubated at 55 C. At various times, samples were chilled and the activity remaining was measured at 30 C.

[b] Determined enzyme activities at 2-h intervals during the early stationary phase (10 to 18 h in BY medium).

[c] Mean values of specific activities in 24- and 30-h cultures (BY medium).

[d] Since the growth rate of *B. natto* 1212 (doubling time, 50 min) was 1.38 times faster than that of *B. subtilis* in BY medium at 30 C, the observed value was normalized with this factor.

[e] Obtained from a cross of *B. natto* 1212 as the donor with strain 6160 as the recipient.

[f] Obtained from a cross of strain Mu8u5u5 (*leu-8 metB5 thr-5 amyEm*$^+$ *amyR1*) as the donor with strain NA20 as recipient (19).

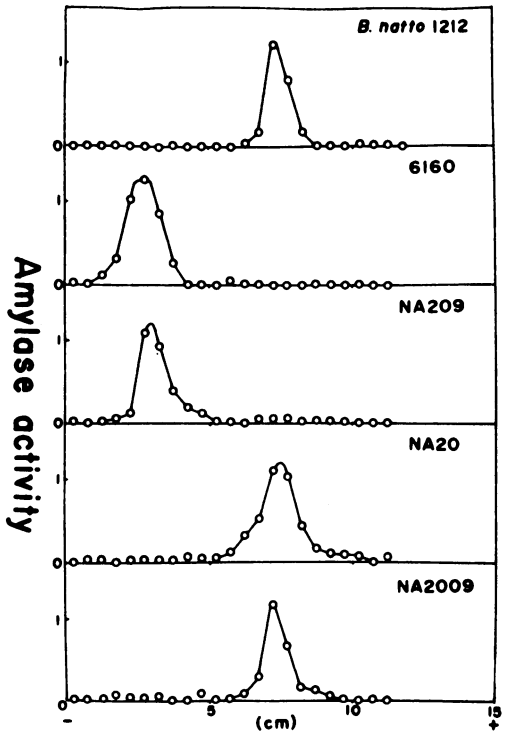

FIG. 1. *Polyacrylamide gel electrophoretic patterns of α-amylases. Extracellular α-amylase was concentrated with ammonium sulfate (90% saturation), dialyzed against tris(hydroxymethyl)aminomethane-glycine buffer (pH 8.3), and mixed with an equal volume of the same buffer containing 20% sucrose. The samples were then applied on polyacrylamide gel columns (0.2 by 15 cm) and subjected to electrophoresis (2 mA per column) for 13 h at 4 C.*

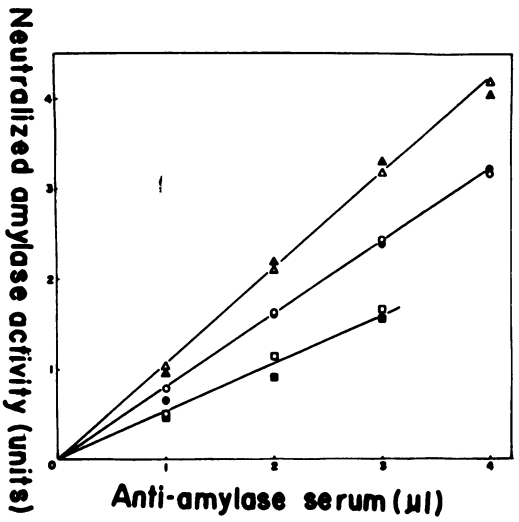

FIG. 2. *Neutralization of α-amylases with anti-amylase serum. Enzyme preparations containing 4.0 to 5.5 U of α-amylase were incubated with various amounts of the antiserum at 40 C for 30 min. The activity remaining was determined. Symbols: ○, 6160; ●, NA209; △, NA2009; ▲, NA20; ■, M9; □, M9-19.*

sizes an α-amylase altered in its thermostability and its immunological properties. Strain M9-19 was an *amyR1* derivative of strain M9, and it produced α-amylase which is not distinguishable from α-amylase (M9). Its rate of enzyme synthesis, however, was fivefold lower than that of strain M9 (Table 1, Fig. 2).

Effect of amyR on the synthesis of CRM. A mutant, M07 (17), derived from strain NA6408 (*amyR2*) is a so-called CRM mutant since it excretes protein that has no α-amylase activity but cross-reacts with anti-amylase serum. Strain 1-131 (*amyEm*$^+$ *amyR1 aro-116*) was transformed by DNA from strain M07, and an *amyE07* derivative was selected from *aro*$^+$ transformants. Fifty colonies picked randomly from among them were cultured in BY medium at 30 C for 24 h, and then CRM activities in the culture fluids were assayed. They were clearly divided into two groups by their CRM activities (Table 3). Group A produced about three times as much CRM as group B. The frequency of group B among *aro*$^+$ transformants was 5.6%, which is close to the expected value of about 4%,

TABLE 2. *Absorption of antibody by α-amylase*[a]

No.	α-Amylase	Antibody (μliters)	Addition	α-amylase activity (U)	
				Observed	Calculated[b]
1	NA209			4.92	
2	NA209	3.5		1.92	
3	NA209	3.5	6160 (2.13 U)	4.29	4.05
4	NA209	3.5	6160 (4.26 U)	6.36	6.18
5	6160			4.71	
6	6160	3.5		1.98	
7	6160	3.5	NA209 (2.16 U)	4.23	4.14
8	6160	3.5	NA209 (4.32 U)	6.19	6.30

[a] After α-amylase (NA209 or 6160) was incubated with anti-amylase serum at 40 C for 30 min and centrifuged at 6,000 × *g* for 15 min, the other α-amylase (6160 or NA209) was added to the supernatant and incubated for an additional 30 min at 40 C. The remaining activity was assayed.

[b] No. 2 (or 6) plus additional α-amylase.

TABLE 3. *Effects of amyR on the CRM synthesis*

aro$^+$ *amyE07* transformants	No.	CRM activity (U/mg of cells)
Group A (*amyR2*)	43	4.54 ± 0.78
Group B (*amyR1*)	7	1.70 ± 0.15

estimated from the distance between *amyR* and *amyE07* (17). Since *amyEm*$^+$ *amyR1* recombinants were obtained from crosses of group B donors with strain NA6408 (*amyEm*$^+$ *amyR2*), we could assign the genotype of group B as *amyE07 amyR1*. The above results show that the rate of CRM synthesis is also regulated by the *amyR* gene.

Specificity of amyR. Whether *amyR* regulates the synthesis of other extracellular enzymes or not is an important question concerning the function of *amyR* gene. *B. natto* 1212 produced a large amount of extracellular protease together with α-amylase (about 400 U and 50 U/mg of cells, respectively). *B. subtilis* 6160 is a low producer of both enzymes (about 45 U and 10 U/mg of cells, respectively). When *B. subtilis* 6160 was transformed by DNA from *B. natto* 1212 and the high α-amylase producers were selected, less than 1% of them produced a large amount of protease simultaneously, indicating that the *amyR* gene is not linked to a gene that concerns protease production. Furthermore, the *amyR* gene was found to have no effect on either the production of extracellular ribonuclease or the doubling time of the organism. Therefore, the action of *amyR* seems to be specific for the α-amylase production.

DISCUSSION

One explanation for the different level of α-amylase activities in *amyR1*- and *amyR2*-carrying strains is that the *amyR* locus might be a part of the structural gene of the enzyme and could effect the specific enzyme activity of α-amylase itself. It was shown, however, that α-amylases of isogenic strains carrying *amyR1* or *amyR2* had biochemically and immunologically identical characteristics regardless of α-amylase type (Table 1 and Fig. 1 and 2). If the higher activity of the *amyR2*-carrying strain arose from the presence of the same amount of altered enzyme molecules having higher specific activity, one could assume that a unit of the antibody should neutralize about five times as much activity of α-amylase of the *amyR2* strain as that of the *amyR1* strain. Figure 2 shows that it is not so. Since after absorption of the antiserum with either α-amylase of *amyR1* or *amyR2* strain there was no antibody remaining to neutralize the other enzyme (Table 2), both enzymes are antigenically similar. The *amyR2* gene also increased the amount of abnormal α-amylase without affecting on its immunological and thermosensitive properties (Table 1 and Fig. 2). Furthermore, the amount of *amyE* product in CRM-producing mutants was found also under the control of *amyR* (Table 3). These results suggest strongly that the introduction of *amyR2* leads to an increase in the amount of *amyE* gene product. The *amyR* gene had apparently no effect on the growth rate of the organism or on the production of other extracellular enzymes such as protease and ribonuclease. Therefore, *amyR* acts specifically on the α-amylase formation.

The *amyR* gene neighbors the structural gene *amyE* according to genetic analysis (17). It is most plausible from our present understanding of the mechanism of gene expression that *amyR* is a promoter site at which the transcription of *amyE* cistron starts. If that is so, one can speculate that *amyR1* in *B. subtilis* Marburg had been derived from *amyR2* by natural mutation events. Promoter mutations in the *lac* operon of *Escherichia coli* can decrease the β-galactosidase level to about 6% of the wild-type level (9). We could not, however, demonstrate the mutation of *amyR2* to *amyR1* in the present study. Recently, Yoneda et al. (18) isolated mutants with the same phenotype as the *amyR2* strain from *amyR1* strains. They produced more of the normal α-amylase than the parents. *amyR2*, an "up" promoter, might be derived from *amyR1*. The I^q mutation of the *lac* operon is located in the *I* gene and involved the stimulation of the synthesis of *I* gene product, i.e., *lac* repressor, to five- to tenfold (8, 10). Fraenkel and Banerjee (3) selected a similar mutation which increases about sixfold the activity of glucose-6-phosphate dehydrogenase in *E. coli*. However, we cannot yet neglect a possibility that the α-amylase synthesis in *B. subtilis* is an inducible one and *amyR* directs a repressor-like product. In this case, *amyR1* but not *amyR2* should code an active repressor-like protein. Further genetic analyses are in progress to elucidate the function of *amyR*.

In our laboratory Yoneda et al. (18) recently found a gene mutation (*pap*$^-$) in *B. subtilis* which stimulates the production of α-amylase and protease simultaneously. The *pap* gene was not linked to the *amyR* gene and is thought to participate in the secretion of the enzymes into extracellular medium. A strain that carries *pap*$^-$ together with *amyR2* produced more α-amylase than a strain that carries only *amyR2*. Ueda and Yuki (15) reported the isolation of α-amylase mutants whose pattern of the enzyme development was somewhat different from that of a wild-type strain. Thus, α-amylase synthesis in *B. subtilis* seems likely to be controlled simultaneously under several genetic loci, including *amyE*, *amyR*, and *pap*.

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