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Isolation of Mutants Defective in α-Amylase from *Bacillus subtilis*: Genetic Analyses

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The rate of α-amylase (EC 3.2.1.1) synthesis in *Bacillus subtilis* is regulated by a gene, *amyR*, located near a structural gene, *amyE*, for the enzyme. To construct a fine map of the *amyR-amyE* region, we isolated 28 mutants defective in α-amylase activity. Eleven mutants out of 28 showed no α-amylase activity, whereas the other 17 showed less α-amylase activity than the parent. Out of 17 partially positive α-amylase mutants, 10 produced temperature-sensitive enzymes, and 4 produced immunologically altered enzymes, two of which are concurrently temperature-sensitive, and 5 produced smaller amounts of α-amylases which are indistinguishable from normal enzyme in their temperature sensitivity and immunological properties. Two out of 11 α-amylase-negative mutants produced material that cross-reacted with anti-amylase serum, and 3 mutants carried suppressible mutations by the suppressor described by Okubo. Mapping data indicate that all 28 mutation sites are located in the *amyE* region, and none of the groups of the mutants mentioned above contains lesions that are clustered in a single region of *amyE*. The *amyR* gene seems most likely to adjoin the terminal region of *amyE*.

Previously (16, 17), it was suggested that at least two genetic elements were involved in α-amylase (EC 3.2.1.1) synthesis in *Bacillus subtilis*. The one, *amyE*, determines the structure of the enzyme, and the other, *amyR*, controls specifically the rate of α-amylase synthesis. The *amyR* is expressed as two genotypes, *amyR1* and *amyR2*. (The genetic symbols of *amyR1* and *amyR2* correspond to $amyR^1$ and $amyR^h$, respectively, that appeared in our earlier papers [16, 18, 19].) The latter was originally found in *B. natto* 1212 and was introduced by a deoxyribonucleic acid-mediated transformation into *B. subtilis* Marburg bearing *amyR1* (16). An organism carrying *amyR2* produces five times more α-amylase than that carrying *amyR1*. However, no experimental evidence has been obtained concerning the action of *amyR*, except that it is closely linked to *amyE*. In this study, we isolated mutants defective in α-amylase activity to obtain further genetic information on the *amyE-amyR* region. α-Amylase-negative mutants of *B. subtilis* had been already isolated by Yuki (22), and their mutation sites were mapped linearly on the chromosome, but it was not clear whether these mutations had occurred in the structural gene or not. Since the *amyR* gene is known to occur near the structural gene *amyE*, it was necessary to confirm by biochemical means that the mutation is in the structural gene.

Among 28 α-amylase-defective mutants isolated, 17 showed less α-amylase activity than the parent and the remainder showed no α-amylase activity. Some of the α-amylase-negative mutants produce material that cross-reacts with anti-amylase serum, and some of them carry suppressible mutations. Results of fine mapping of 28 markers, together with biochemical evidence, indicated that all of the mutations occurred within the region of *amyE* and that *amyR* adjoins a terminal region of *amyE*.

MATERIALS AND METHODS

Bacterial strains and bacteriophages. The strains of *B. subtilis* used are listed in Table 1. They are derived from strain 168 of Burkholder and Giles (3). Bacteriophage SPO1 wild type and its suppressor-sensitive mutants *sus8* and *sus31* were kindly supplied by S. Okubo. Phage suspensions were prepared from confluent lysed areas with strain HA101B as a host.

Isolation of α-amylase-defective mutants by ultraviolet irradiation. Spores of strain NA64 were

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TABLE 1. *List of strains used*

Strain[a]	Genotype	Origin
6160	*metB5 purB6 trp-160 amyE⁺ amyR1*	Y. Ikeda
1-131	*met his aro-116 amyE⁺ amyR1*	S. Yuki
NA64	*metB5 purB6 amyE⁺ amyR2*	From strain 6160 by transformation from *B. natto* 1212 (16)
NA6408	*metB5 aro-116 amyE⁺ amyR2*	From strain NA64 by transformation from strain 1-131
HA101	*met his leu amyE⁺ amyR1*	Okubo and Yanagida (9)
HA101B	*met his leu sup amyE⁺ amyR1*	Okubo and Yanagida (9)
HA101-1	*met his aro-116 amyE⁺ amyR1*	From strain HA101 by transformation from strain 1-131

[a] The strains are derivatives of *B. subtilis* 168 (3).

prepared by using Scheaffer medium (20) and then diluted to a concentration of about 10^8/ml in 0.8% NaCl solution. After irradiation with an ultraviolet lamp (Toshiba GL-15) to a survival of 5×10^{-5}, spores were incubated for 3 h in bouillon-yeast extract (BY) medium (5) at 37 C and then plated on BY agar plates containing 1% soluble starch. When spores of strain 1-131 were irradiated, 0.2% soluble starch was used to detect mutants. After incubation overnight at 45 C, the plates were replicated to another BY agar plate and then 0.01 M I_2-KI solution was sprayed onto the master plate. Large unstained halos were observed around wild-type colonies, but only a small or no halo was observed around colonies of the mutants.

Isolation of mutants by N-methyl-N'-nitro-N-nitrosoguanidine treatment. Exponentially growing cells were incubated with 100 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine per ml for 15 min at 37 C in tris(hydroxymethyl)aminomethane-malate buffer (pH 6.0) as described by Adelberg et al. (1). Treated cells were collected on a membrane filter (HA type; Millipore Corp.), washed with the same buffer, and grown at 37 C for 3 h in BY medium. Detection of mutants was the same as in the case of ultraviolet irradiation method.

Procedure of transformation. Transforming deoxyribonucleic acid was extracted from donor strains by using phenol saturated with 0.1 M tris(hydroxymethyl)aminomethane buffer (pH 9.0) containing 1% sodium lauryl sulfate (11). Transformation experiments were performed as described by Saito et al. (10). The deoxyribonucleic acid was used at a concentration of 0.1 μg/ml. Since *aro-116* was known to be a linked marker of *amyE* and *amyR*, *aro*⁺ transformants were selected on minimal medium (14) agar plates supplemented with 1% soluble starch and all requirements except aromatic amino acids, and tested for productivity of α-amylase. When mutant M12 or M18 was used as a donor, *aro*⁺ transformants were restreaked on 1% soluble starch-BY agar plates and then tested for the enzyme level after incubation overnight at 43 and 48 C, respectively.

Culture conditions and preparation of α-amylase. Bacteria were grown overnight with aeration in BY medium at 30 C, and the culture was diluted 100-fold with fresh BY medium. The organisms except for strain M12 were cultivated further at 30 C for 20 to 28 h until the maximal enzyme activity was attained. Cultivation of strain M12 was stopped at 13 h because its α-amylase activity rapidly decreased during the stationary growth phase. 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 α-amylase activity in the crude enzyme preparation was less than 5 U/ml, the enzyme solution was concentrated by the addition of ammonium sulfate to 90% saturation. The precipitate dissolved in 0.04 M phosphate buffer (pH 6.0) was extensively dialyzed against the same buffer at 4 C.

Assay of α-amylase 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. To determine the thermostability of α-amylases, the enzyme solution (7 to 10 U) was incubated at 55 C for 20 min. At various times, samples were chilled and the enzyme activity remaining was measured at 30 C.

Preparation of anti-amylase serum. The antiserum was prepared with α-amylase of *B. subtilis* var. *amylosachariticus* (Seikagaku Kogyo Co.) as antigen (17).

Neutralization of α-amylase activity with anti-amylase serum and determination of amounts of CRM. A 0.5-ml volume of the enzyme solution was incubated with the same volume of the antiserum at 40 C for 30 min, and the remaining activity was assayed. The antiserum was diluted appropriately with 0.85% NaCl solution. To determine the amount of cross-reacting material (CRM), a known amount of the indicator α-amylase was mixed with various amounts of a culture fluid of a given strain, and then the antiserum was added. The reaction mixture was incubated at 40 C for 30 min. One unit of CRM was defined as the amount of material that was antigenically equivalent to 1 U of α-amylase.

RESULTS

Isolation of α-amylase mutants. To perform simultaneous isolation of mutants negative or temperature sensitive in α-amylase activity, detection was carried out at 45 C (see above).

The activity level of α-amylase and some properties of 28 mutants were summarized in Table 2. The α-amylases of these mutant strains were studied in detail as follows.

Temperature-sensitive α-amylases. The fourth column in Table 2 shows the thermostability of α-amylase produced by partially positive mutants. The temperature-sensitive α-amylases were classified into three groups in terms of their half-lives at 55 C: (i) 0.2 min (M12), (ii) 2 to 3 min (M9, M20, and M21), and (iii) 10 to 20 min (M1, M3, M13, M14, M18, and M011). Heat-inactivation curves of α-amylases (M1014, Mu1, M10, M01, M06, M010, and M016) were similar to that of the wild-type enzyme, and their half-lives were between 35 and 45 min. From genetic studies on the temperature-sensitive α-amylase-producing mutants, their mutation sites were found to be distributed throughout the *amyE* region (see Fig. 4).

Immunologically altered α-amylases. α-Amylases of partially positive mutants were mixd with anti-amylase serum, and the degree of neutralization was examined (Table 3). Apparently, mutants Mu1, M1, M9, and perhaps M06 synthesized immunologically altered enzymes. The other mutants produced immunologically indistinguishable α-amylase from the normal one, but in smaller amounts.

CRM-producing mutants. When a known amount of α-amylase (NA64) was mixed with a culture fluid of strain M07 or M1010 and then

TABLE 2. *Properties of α-amylase-defective mutants*

Strain	Origin	Level of α-amylase[a] (U/mg of cells)	Thermostability of α-amylase (half-life at 55 C [min])	Summary
Parents				
NA64		53.8	36 ± 3	Normal
1-131		9.7	32 ± 3	Normal
Mutants[b]				
M1001	1-131	<0.1		Negative (*sus*)
M1003		<0.1		Negative
M1010		<0.1		Negative (CRM)
M1014		3.9	45 ± 5	
Mu1	NA64	0.78	40 ± 5	IM
M1		0.62	12 ± 1	TS, IM
M3		7.9	13 ± 1	TS
M9		7.5	2.0 ± 0.2	TS, IM
M10		0.31	34 ± 5	
M11		<0.1		Negative (*sus*)
M12		1.2	0.2	TS
M13		8.1	11 ± 1	TS
M14		8.0	10 ± 1	TS
M15		<0.1		Negative (deletion)
M17		<0.1		Negative
M18		26.4	12 ± 1	TS
M20		9.6	2.9 ± 0.2	TS
M21		10.0	1.9 ± 0.2	TS
M01	NA6408	0.76	34 ± 4	
M03		<0.1		Negative
M05		<0.1		Negative (*sus*)
M06		0.71	40 ± 5	IM
M07		<0.1		Negative (CRM)
M08		<0.1		Negative
M010		0.49	36 ± 4	
M011		1.9	21 ± 1	TS
M016		2.6	45 ± 5	
M017		<0.1		Negative

[a] Mean values of specific activities of 20- and 26-h cultures (BY medium) at 30 C.

[b] Only strain Mu1 was induced by an ultraviolet irradiation method; the others were induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment.

TABLE 3. *Neutralization of α-amylases with antiserum*[a]

Expt I			Expt II[b]		
α-Amylase source	Neutralized activity with 1 μliter of antiserum		α-Amylase source	Neutralized activity with 1 μliter of antiserum	
	U	%		U	%
NA64	1.45 ± 0.20	100	NA64	0.94 ± 0.08	100
M9	0.43	30	M1014	1.01	106
M13	1.43	99	M1	0.07	7
M14	1.58	109	M10	1.01	106
M18	1.27	88	Mu1	0.00	0
M20	1.19	82	M01	0.83	87
M21	1.18	81	M06	0.69	73
			M010	0.13	119
			M011	0.88	93
			M016	1.03	108

[a] Neutralization of α-amylase (4 to 7 U) was examined with various amounts of antiserum (0.5 to 3 μliters), and neutralized activity with 1 μliter of antiserum was determined. Values presented were the average of two or three experiments.

[b] The titer of the antiserum used in experiment II had decreased during the storage for 10 months.

anti-amylase serum was added, neutralization of the indicator α-amylase was inhibited. The neutralization curve of α-amylase (NA64) in the presence of the culture fluid of strain M07 (Fig. 1) shows that 0.3 ml of strain M07 culture fluid contained an amount of CRM sufficient to neutralize 1 U of anti-amylase serum. Strain M1010 showed lower but significant neutralization inhibition activity. No CRM activity was detected in the culture fluids of the other α-amylase-negative mutants. The CRM activity of strain M07 carrying *amyR2* was 6.07 U/mg of cells, whereas that of strain M1010 bearing *amyR1* was 1.44 U/mg of cells.

Suppressor-sensitive mutants. Nonsense mutations are suppressed by a suppressor gene that leads to the insertion of a specific amino acid into a polypeptide chain that otherwise would be an inactive protein (2, 13, 15). Suppressor mutants of *B. subtilis* have been isolated by Okubo and Yanagida (9) and Georgopoulos (4), who obtained *sus* mutants of bacteriophage SPO1 and Øe, respectively.

We tried to suppress α-amylase-negative mutants with the suppressor of Okubo. The suppressor mutation in strain HA101B (*met his leu sup*) derived from strain HA101 (*met his leu*) can suppress simultaneously both *met* and *his*. First, the α-amylase-negative character was introduced into strain HA101 by transformation, and next the resultant *met his amy* strains were received as *sup* from strain HA101B. Phenotypic Met$^+$ His$^+$ double transformants could be isolated at almost the same frequency (10^{-3}) as single transformants. When strains HA1001 (*amyE1001 met his*), HA11 (*amyE11 met his*), and HA05 (*amyE05 met his*) were used as recipients, all 20 Met$^+$ His$^+$ "double" transformants were phenotypically α-amylase positive and all of the double transformants obtained from other crosses were α-amylase negative. Strains HA1001B, HA11B, and HA05B were Met$^+$ His$^+$ Amy$^+$ transformants showing 10 to 15% of the α-amylase activity of the wild-type strain (Table 4). The susceptibility of these strains to bacteriophage SPO1 *sus* mutants was investigated to prove that the transformants were really *sup*. Since mutants *sus8* and *sus31* of phage SPO1, which had been isolated by Okubo and Yanagida (9), could form plaques on these three strains as they did on strain HA101B, it was confirmed that they carried the suppressor. Furthermore, the genotype of these three strains should be *amy*, since 38 to 42% of *aro*$^+$ transformants were simultaneously *amy* when strain 1-131 (*amyE*$^+$ *aro-116*) was transformed by deoxyribonucleic acid from strains HA1001B, HA11B, or HA05B.

These results lead us to conclude that *amyE1001*, *amyE11*, and *amyE05* were suppressible by Okubo's suppressor, resulting in

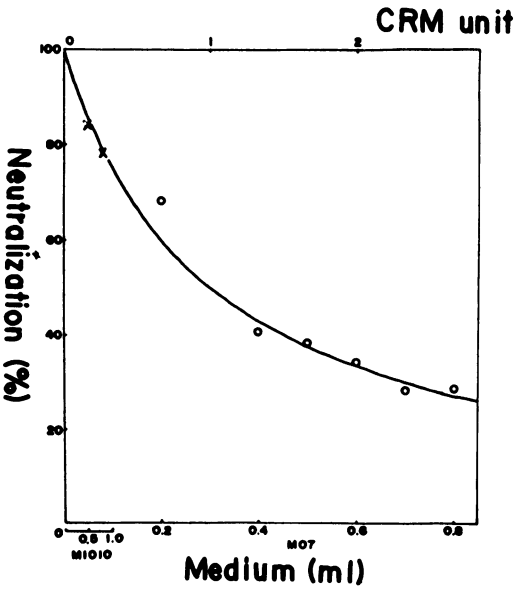


FIG. 1. *Titration of CRM in the culture fluids of strains M07 and M1010. Values are expressed as percentage of control in which no culture fluid was added. Symbols:* O, *M07;* ×, *M1010.*

the synthesis of partially active α-amylase. In a preliminary experiment, α-amylase (HA11B) could be distinguished from the normal enzyme in its thermostability, since the suppression would result in the insertion of an amino acid different from that of normal α-amylase.

Fine mapping of the amyR-amyE region. Every *amy* marker isolated in the present study was co-transformable with *aro-116* at a frequency of 30 to 40%. Therefore, the different competency of recipient was not necessarily normalized in three-point transformation experiments with *aro*$^+$ as a selected marker (8). Recombination frequency between two *amy* markers was expressed as the percentage of *amy*$^+$ recombinants among *aro*$^+$ transformants (Table 5). Since only five strains were used as recipients, not all the markers could be mapped exactly, but a relative order of eight groups could be assigned (Fig. 2). Whenever *amyE1010* was used as a donor marker, a low frequency of recombination was observed. The marker *amyE1010* might be a short-range deletion mutation since the recombination frequency was known to be dependent on the length of deletion mutation in the amylomaltose gene of *Pneumococcus* (6). Strain M15 could be a deletion mutant of *amyE* region since no *amy*$^+$ recombinant could be detected in crosses with any recipients. In the cross of donor strain M15 with strain 1-131 (*amyE*$^+$ *amyR1 aro-116*), all the *aro*$^+$ *amyE*$^+$ transformants (1,200 colonies) bore *amyR1*. Therefore, *amyE15* should be a deletion of not only *amyE* but also *amyR*.

In three-point cross experiments, two possible configurations can be considered (Fig. 3). Supposing that A and B are defective sites in *amy*, a frequency of *amy*$^+$ recombinants among the *aro*$^+$ transformants should be higher in case of configuration I than in configuration II, since in

TABLE 4. *Suppression of amyE1001, amyE11, and amyE05 by a suppressor*

Strain	α-Amylase activity[a] (U/mg of cells)
HA1001 (*amyE1001 sup*$^+$)	<0.05
HA1001B (*amyE1001 sup*)	0.76
HA11 (*amyE11 sup*$^+$)	<0.05
HA11B (*amyE11 sup*)	0.64
HA05 (*amyE05 sup*$^+$)	<0.05
HA05B (*amyE05 sup*)	1.57
HA101-1 (*amyE*$^+$ *sup*$^+$)	7.8
HA101B (*amyE*$^+$ *sup*)	11.7

[a] Specific activity of 24-h culture (BY medium) at 30 C.

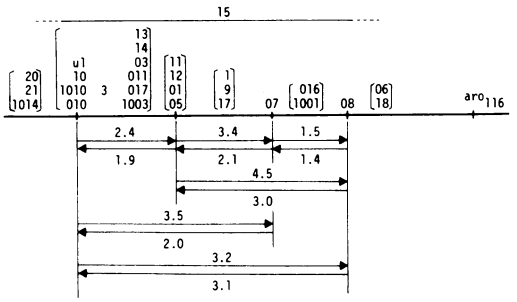


FIG. 2. *Marker order assigned from the recombination data and the reciprocal three-point transformation crosses between amy mutants. The recipient strain has a mutation indicated at an arrowhead.*

TABLE 5. *Recombination data in three-point transformation crosses*

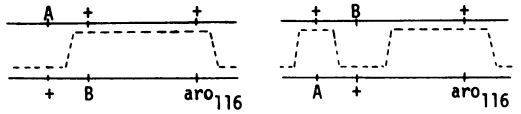
Recipient	Percentage[a] of *amy*$^+$ with donors:													
	21	20	1014	1010	010	10	ul	3	14	03	011	017	1003	13
08	3.6	2.8	3.6	1.3	3.2	3.2	2.8	2.6	2.8	3.3	3.3	2.5	2.2	2.9
07	4.1	3.7	4.1	0.6	3.5	2.9	1.9	1.3	2.2	2.2	2.0	2.3	1.7	1.6
01	2.7	3.1			2.1	1.9	1.8	1.4	1.1	1.0		1.2		1.0
05	2.9	2.1	2.6	0.3	2.4						1.4	1.9		
010	2.0	1.9	1.7	0.1	<0.01					0.5	0.5	0.7	0.7	

Recipient	Percentage[a] of *amy*$^+$ with donors:													
	05	01	12	11	17	9	1	07	1001	016	08	18	06	15
08	4.5	1.3	2.2	1.8	1.4	2.6	1.7	1.5	0.8	0.4	<0.02	0.4	0.9	<0.04
07	3.4	1.3	1.6	1.7	0.9	0.7	0.4	<0.01	0.4	0.5	1.4	1.4	1.5	<0.01
01	0.6	<0.01	0.6	0.6	1.0									
05	<0.01	0.5		1.2	1.6			2.1			3.0	2.4	2.5	<0.01
010	1.9			2.0	1.7		1.9	2.0	2.0	1.6	3.1	1.6		<0.04

[a] Percentage of *amy*$^+$ in *aro*$^+$ transformants; 10^3 to 10^4 *aro*$^+$ transformants were analyzed in each cross.

the latter case at least four crossover events are necessary for appearance of *amy*$^+$ recombinant. Reciprocal three-point crosses were carried out (Table 5, Fig. 2). Recombination values in crosses where a mutation site of a donor is on the left of a recipient site were always higher than the values where a donor site is on the right of a recipient site. These results suggest that *aro-116* is located on the right of *amyE08*.

Location of amyR. The relative location of *amyR* to *amyE* markers was determined by genetic crosses between *amyE* mutants with *amyR1* or *amyR2*. In case of cross I in Table 6, when *amyR* is located in the *amyE1001* distal side of the donor marker *amyE010*, most of *amy*$^+$ recombinants would be *amyR1*, whereas a large portion of *amy*$^+$ would be *amyR2* if *amyR* is located in the *amyE1001* proximal side of a donor marker. The results indicated the former case. Data from other crosses also suggest that *amyR* could be mapped to the left of *amyE*. The order of *amyR-amyE1014-amyE010-(amyE-1010, amyE1003)-amyE08-aro-116* was concluded from the data presented in Fig. 2. Therefore, the order *amyR-amyE-aro-116*, proposed by Yuki (21), was confirmed.



Configuration I Configuration II

FIG. 3. *Possible configurations when amy*$^+$ *recombinants were determined among aro*$^+$ *transformants.*

It was presumed that *amyE20* and *amyE21*, among 28 markers, were situated nearest the *amyR* gene (Fig. 2, Table 6). The experiment in Table 7 was performed to calculate the recombination frequencies between *amyR* and these markers. Mutants M20, M21, and M010, used in cross I as donors, were partially positive in α-amylase activity. After a spraying with I_2-KI solution, transparent halos were detected around the colonies of strains M20 and M21 grown at 30 C and of strain M010 grown at 45 C on the 0.2% soluble starch-BY agar plate. The *amyR1* recombinants from these mutants would have less α-amylase activity. Actually, 1.5% of *aro*$^+$ transformants in cross I-3 showed small red halos around their colonies. Since *amyE*$^+$ *amyR1* recombinants were isolated from the transformation of strain NA64 (*amyE*$^+$ *amyR2*) with deoxyribonucleic acid from one of these

TABLE 6. *Location of amyR: four-point transformation crosses*

Cross	Donor genotype[a]	Recipient genotype[a]	No. of *amy*$^+$*aro*$^+$ recombinants		Probable order
			amyR2	*amyR1*	
1	*010 amyR2*	*1001 amyR1 aro-116*	4	26	*amyR-010-1001*
2	*1001 amyR1*	*010 amyR2 aro-116*	0	7	*amyR-010-1001*
3	*1003 amyR1*	*08 amyR2 aro-116*	36	4	*amyR-1003-08*
4	*1010 amyR1*	*08 amyR2 aro-116*	31	5	*amyR-1010-08*
5	*1014 amyR1*	*010 amyR2 aro-116*	14	1	*amyR-1014-010*
6	*1003 amyR1*	*010 amyR2 aro-116*	3	5	*amyR-010-1003*
7	*1010 amyR1*	*010 amyR2 aro-116*	1	2	*amyR-010-1010*
8	*1014 amyR1*	*05 amyR2 aro-116*	9	1	*amyR-1014-05*

[a] Markers of *amyE* were expressed just by numbers.

TABLE 7. *Recombination frequency between amyR and amyE20, amyE21, or amyE010*

Donor genotype	Recipient genotype	No. of recombinants	
		amyE20, amyE21 or *amyE010 amyR1/aro*$^+$	*amyE*$^+$ *amyR2/aro*$^+$
Cross I			
1. *amyE20 amyR2*	*amyE1001 amyR1 aro-116*	0/1257 (<0.08%)	
2. *amyE21 amyR2*	*amyE1001 amyR1 aro-116*	0/1260 (<0.08%)	
3. *amyE010 amyR2*	*amyE1001 amyR1 aro-116*	19/1258 (1.5%)	
Cross II			
1. *amyE*$^+$ *amyR1*	*amyE20 amyR2 aro-116*		0/1200 (<0.08%)
2. *amyE*$^+$ *amyR1*	*amyE21 amyR2 aro-116*		0/1244 (<0.08%)
3. *amyE*$^+$ *amyR1*	*amyE010 amyR2 aro-116*		31/1200 (2.6%)

It was presumed that *amyE20* and *amyE21*, among 28 markers, were situated nearest the *amyR* gene (Fig. 2, Table 6). The experiment in Table 7 was performed to calculate the recombination frequencies between *amyR* and these markers. Mutants M20, M21, and M010, used in cross I as donors, were partially positive in α-amylase activity. After a spraying with I_2-KI solution, transparent halos were detected around the colonies of strains M20 and M21 grown at 30 C and of strain M010 grown at 45 C on the 0.2% soluble starch-BY agar plate. The *amyR1* recombinants from these mutants would have less α-amylase activity. Actually, 1.5% of *aro*$^+$ transformants in cross I-3 showed small red halos around their colonies. Since *amyE*$^+$ *amyR1* recombinants were isolated from the transformation of strain NA64 (*amyE*$^+$ *amyR2*) with deoxyribonucleic acid from one of these

transformants, it was concluded that the genotype of transformants was *amyE010 amyR1*. On the other hand, *amyR1* recombinants carrying *amyE20* or *amyE21* could not be detected among *aro*$^+$ transformants (crosses I-1 and 2). These results were confirmed further by cross II experiment. In this case, recombinants were detected as *amyE*$^+$ *amyR2*, and the recombination frequency between *amyR* and *amyE20* or *amyE21* was less than 0.08% of *aro*$^+$ transformants. The α-amylase level of strains M20 and M21 grown at 30 C was almost the same as that of strain 6160 (*amyE*$^+$ *amyR1*), and it seems as if their *amyR2* mutated to *amyR1*. However, α-amylase of strains M20 and M21 could be distinguished from the normal enzyme in their thermostability (Table 2) and electrophoretic mobility (data not shown). It is deduced that both strains M20 and M21 are defective in the structural gene of α-amylase, *amyE*.

When *aro*$^+$ strains (1,200 colonies) were prepared from a cross of donor strain NA64 (*amyE*$^+$ *amyR2*) with strain 1-131 (*amyE*$^+$ *amyR1 aro-116*) and the productivity of α-amylase was judged on 0.2 and 1% soluble starch-BY agar plates, the level of α-amylase productivity of each *aro*$^+$ transformant was either donor type (*amyR2*) or recipient type (*amyR1*), and no recombinant producing another level of α-amylase could be detected.

DISCUSSION

Characterization of α-amylase-defective mutants. Twenty-eight mutants defective in α-amylase activity were isolated. Seventeen out of 28 mutants produced less α-amylase, whereas the other 11 mutants showed no α-amylase activity. Of 17 partially positive α-amylase mutants, 10 produced temperature-sensitive α-amylases, 4 produced immunologically altered α-amylases, two of which are simultaneously temperature sensitive, and 5 produced less amounts of α-amylases which are indistinguishable from normal enzyme with respect to their temperature sensitivity and immunological property. Two out of 11 α-amylase-negative mutants are CRM mutants, and 3 carry suppressible mutations (Table 2, last column).

The immunologically altered α-amylases might be changed in their binding specificity to the antibody or in their efficiency of the enzyme action. The difference of CRM activity between mutants M07 (*amyR2*) and M1010 (*amyR1*) supports the idea that the synthesis of CRM is also controlled under the *amyR* gene as reported in the preceding paper (17). Markers *amyE05*, *amyE11*, and *amyE1001* were suppressed by the suppressor gene found by Okubo and Yanagida (9). They are the first example of the effect of *sus* mutation of a well-identified protein like α-amylase in *B. subtilis*, although many *sus* mutants from bacteriophage SPO1 and Øe had been isolated (4, 9). The levels of enzyme activity in suppressed mutants were about 10 to 15% of that of the wild-type strain (Table 4). These values, however, did not show the same degree of suppression, since it is plausible that the suppressed enzyme differs from normal α-amylase as observed in suppressed α-amylase (HA11B).

Mutants M1014, M10, M01, M010, and M016 showed lower α-amylase activity, and their mutation sites were mapped within the *amyE* region (Table 2 and Fig. 4). It is possible that the rate of α-amylase production decreased in these mutants, since no detectable difference was observed between α-amylase of a parent strain and that of the mutant strains. One interpretation could be Stent's modulation theory (12): mutated codons become complementary to minor transfer ribonucleic acids, resulting in its concentration in cells becoming a rate-limiting factor in protein synthesis. The other interpretation could be a sort of feedback regulation: the rate-limiting step in α-amylase production is the secretion step, and even a minor conformational change in a mutated α-amylase causes change of its secretion rate, resulting in a certain level of intracellular α-amylase repressing the synthesis of the protein. It might support the latter interpretation since the amounts of the cell-bound enzyme in these mutants were almost the same as in a wild strain, and no mutant overproducing cell-bound α-amylase was isolated in the present study (7).

Genetic map of amyR-amyE region. All the *amy* mutations listed in Table 2 were co-transferred with *aro-116* at a frequency of 30 to 40%. Thus, the genetic map in Fig. 4 was tentatively constructed with *aro-116* as a selected marker from the results of three-point and four-point transformation crosses. The recombination frequencies between markers tested were usually additive, especially within small regions. The figure shows that the mutations occurring at the *amyR* proximal and distal end, i.e., *amyE20* (or *amyE21*) and *amyE06*, caused the synthesis of a biochemically altered α-amylase, and, since all the other mutations could be mapped between *amyE20* (or *amyE21*) and *amyE06*, all the mutants isolated in the present study should be *amyE* mutants. Therefore, 10 markers, *amyE1003*, *amyE1014*, *amyE10*, *amyE17*,

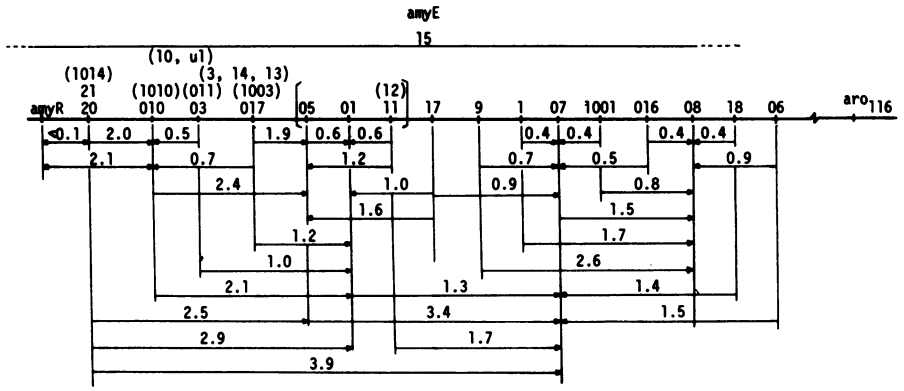


FIG. 4. *Tentative genetic map of amyE markers from transformation data. The values are expressed as percentage of amy⁺ recombinants per aro⁺ transformants obtained from a cross where a recipient strain has a mutation indicated at an arrowhead. Parentheses imply that a mutation site was not yet definitely established in the present study. The order of markers in a bracket was not yet assigned from these transformation data. In this figure, recombination values obtained from crosses where a mutation site in a donor strain is on the left of the recipient-site (Configuration I in Fig. 3) are presented.*

amyE01, *amyE03*, *amyE08*, *amyE010*, *amyE016*, and *amyE017*, which could not be identified as mutations in the structural gene by biochemical means were mapped within the *amyE* region. It should be noted that ten temperature-sensitive mutations in α-amylase were distributed all over the *amyE* gene, and the same was true for four immunologically altered mutations, three *sus* mutations, and two CRM mutations.

The recombination frequencies between *amyR* and a marker (*amyE20* or *amyE21*) located at the *amyR* proximal side of *amyE* were less than 0.08%. The markers should, however, be situated in *amyE* since both mutations directed the synthesis of temperature-sensitive α-amylases. The molecular weight of α-amylase (NA64) has been determined to be about 55,000 (18); it can therefore be estimated that *amyE* is about 1,300 base pairs long. Because the recombination frequency between *amyE20* (or *amyE21*) and *amyE06* situated at the both ends of *amyE* was 4 to 10%, the distance between *amyE20* (or *amyE21*) and *amyR* would be calculated to be 10 to 30 base pairs or less. Although one cannot estimate precisely the true distance from the data of recombination frequency, the region between the *amyR* gene and the *amyE* gene seems much smaller than the average gene. We would conclude that *amyR* adjoins to or occupies a terminal region of the structural gene *amyE*.

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