GENETIC STUDIES ON AMYLASE OF DIFFERENT ELECTROPHORETIC MOBILITY PRODUCED BY STRAINS OF *BACILLUS SUBTILIS*

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Amylase of *Bacillus subtilis* is an extra-cellular enzyme, whose production has unique characteristics and is different from that of ordinary intra-cellular enzymes extensively investigated in bacterial genetics. The amylase of *B. subtilis* is known to be produced at the later phase of bacterial growth cycle; *i.e.* after cells stop their growth. The amylase activity is detectable in the culture medium but not within the bacterial cells. The amylase is assumed to be produced from an inactive intermediate product (Nomura *et al.* 1958, and Yoshida *et al.* 1960), though the mechanism of its activation and secretion still remains obscure.

In spite of the fact that biochemical studies have extensively been carried out on the amylase production in *B. subtilis*, very little information is available on the genetic mechanism of its production. Green *et al.* (1964) studied the transformation of a strain of *B. subtilis* by DNA obtained from another strain whose amylase was electrophoretically and enzymatically different from that of the former strain. From the results of this experiment, they concluded that genes controlling the production of amylases of the respective strains were not allelic.

In the present study, the genetic analyses on the amylase genes of different origins which control amylases with different electrophoretic mobilities were undertaken by DNA transformation, using the amylase negative mutants as recipients.

MATERIALS AND METHODS

BACTERIA The strains used to isolate amylase negative mutants were *Bacillus subtilis* 168, designated as 1-168 (try_2^-), and its derivatives, 1-49 ($try_2^- his_1^- cys^- sm^r$) and 1-74 ($his_1^- cys^- sm^r$). 1-18 (his_2^-), another derivative of 1-168, was used as the donor in the transformation experiment. Other strains of *B. subtilis*, 2-23, 31, 37, 38, 44, 45, 46, 90, 131, 149, 158, 183, 186, 189 and 195, were genealogically different strains from the 1-168 strain, but their DNAs were able to transform 1-168 at frequencies comparable to that of homologous DNA. 2-7 was an amylase negative mutant of 2-23. The 1088 strain (Green *et al.* 1964) was obtained from Dr. D. M. Green of Pittsburgh University.

MEDIA The culture media used in this study were as follows: *Nutrient broth*; meat extract 10 g, polypeptone 10 g and NaCl 2 g in 1000 ml of water, adjusted to pH 7.0 by 10 N NaOH before autoclaving. *Minimal medium*; K_2HPO_4 14 g, KH_2PO_4 6 g, $MgSO_4 \cdot$

7H₂O 0.2 g, (NH₄)₂SO₄ 2 g, Na-citrate·2H₂O 1 g and glucose 5 g in 1000 ml of distilled water. *CHT-2*; minimal medium containing 0.02 % casamino acid and 20 µg/ml of DL-tryptophan. *CHT-10*; minimal medium containing 0.1 % casamino acid and 200 µg/ml of DL-tryptophan. *Potato agar*; 200 g of peeled, chipped potatoes were boiled in one liter of tap water, and the boiled potatoes were filtered through several sheets of gauze. After the addition of 5 mg of MnSO₄ to the filtrate, the pH was adjusted to 6.8. The volume was then made to one liter by adding tap water.

A solid medium was prepared by adding 15 g of agar per liter of liquid medium. When required, 10 µg/ml of amino acid was supplemented to the minimal medium.

METHOD FOR ISOLATING TRANSFORMING DNA Transforming DNA was extracted according to the method of Marmur (1961). Deproteinization by chloroform was done twice. RNase treatment and the subsequent procedures were omitted from the method.

PROCEDURE OF TRANSFORMATION The transformation experiment was performed according to the method of Nester *et al.* (1961). A 0.5 ml of overnight culture of the recipient cell was centrifuged and suspended in 5 ml of CHT-2. After cultivation with shaking for 4 hours at 37°C, the cell culture was diluted to one tenth of cell density in CHT-10 and cultivated for another 1.5 hours. DNA was then added to the cell culture and the mixture was incubated with gentle shaking for 30 minutes. After washing with minimal medium without glucose, a 0.1 ml of appropriately diluted suspension was plated on the selective plate. Transformants which thus appeared were scored after two overnight incubations at 37°C.

ASSAY OF AMYLASE ACTIVITY Activity of amylase was assayed according to the method described by Fuwa (1954). As a substrate, soluble starch was used instead of amylose in the method. Decrease of absorbancy at 660 mµ was used as the relative amylase activity.

AGAR GEL ELECTROPHORESIS Agar gel electrophoresis was performed according to the method described by Ogita (1963). The agar gel contained 0.7 g of agar and 0.5 g of CHC (cellosize hydroxylcellulose) in 100 ml of 0.0125 M phosphate buffer (pH 5.8). In a later period of this study, 0.7 g of PVP (polyvinyl pyrrolidon) was used instead of CHC. CHC and PVP added to minimize electro-endosmotic flow produced similar results except that the latter somewhat interfered with coloration by iodostarch reaction. Agar gel melted in boiling water was filtered with 16 sheets of gauze and a thin layer was made on a glass plate in the size of 16.2×11.8 cm² at the thickness of 0.9 mm.

Supernatant of 24 hour culture of each strain in nutrient broth was used as an enzyme sample without further concentration. The enzyme sample was adsorbed to a filter paper strip (1×10 mm²) and the strip was placed on the agar gel along the original line which is 6.5 cm from the cathode side, and the enzyme was allowed to diffuse into the agar gel for 20 to 30 minutes at 5°C. After the filter paper strip was carefully taken off, the enzyme was electrophoresed. Voltage across the gel was 200 volts. After a 90 minute electrophoresis, the agar gel was immersed in a 1 % starch solution

in buffer for 5 minutes and incubated for 60 to 90 minutes at 37°C. Then, a 0.1 N iodine solution was overflowed on the agar gel. By this procedure, the portion where amylase was present appeared as a color-less band against a bluish background.

RESULTS

ISOLATION OF AMYLASE NEGATIVE MUTANTS. An attempt was performed to isolate amylase negative mutants (*amy*⁻) from 1-168, 1-49 and 1-74. The isolation of *amy*⁻ mutants could barely be achieved by irradiating vegetative cells by ultraviolet light, but it was rather easily achieved by irradiating spores. Spores of 1-168, 1-49 and 1-74 formed after more than three day incubation of the cells on potato agar were suspended in a minimal medium without glucose. Then, 5 ml of the suspension (approximately 10⁸ spores/ml) in a sterile petri dish (9 cm in diameter) was irradiated by a germicidal lamp (GL 10, Toshiba) for 12 minutes. The survival frequency was approximately 10⁻³. The irradiated spores were spread on nutrient agar plates containing 0.2 % of soluble starch and incubated at 37°C to form colonies. After overnight incubation, an iodine solution (0.1 N) was flooded on the colonies and the colonies were searched for *amy*⁻ colonies. The original *amy*⁺ colony showed an unstained halo of hydrolysed starch around it, while the *amy*⁻ colony when it appeared, did not show such a halo. By this method, *amy*⁻ mutants were isolated at the frequency of approximately 5×10⁻⁴ of surviving spores. Twelve *amy*⁻ mutants thus obtained were named A1 through A12 and some of their properties so far studied are listed in Table 1.

Table 1. Characteristics of amylase negative mutants and the original strains

Strain	Origin	Genetic marker	Colony	Sporulation
A1	1-168	<i>try</i> ₂ ⁻	dry	+
A2	1-168	<i>try</i> ₂ ⁻	dry	+
A3	1-168	<i>try</i> ₂ ⁻	soft	-
A4	1-49	<i>try</i> ₂ ⁻ <i>his</i> ₁ ⁻ <i>cys</i> ⁻ <i>sm</i> ^r	dry	+
A5	1-74	<i>his</i> ₁ ⁻ <i>cys</i> ⁻ <i>sm</i> ^r	dry	+
A6	1-168	<i>try</i> ₂ ⁻	dry	+
A7	1-168	<i>try</i> ₂ ⁻	dry	+
A8	1-168	<i>try</i> ₂ ⁻	dry	+
A9	1-168	<i>try</i> ₂ ⁻	dry	-
A10	1-168	<i>try</i> ₂ ⁻	dry	+
A11	1-168	<i>try</i> ₂ ⁻	soft	+
A12	1-168	<i>try</i> ₂ ⁻	dry	+
1-168		<i>try</i> ₂ ⁻	dry	+
1-49		<i>try</i> ₂ ⁻ <i>his</i> ₁ ⁻ <i>cys</i> ⁻ <i>sm</i> ^r	dry	+
1-74		<i>his</i> ₁ ⁻ <i>cys</i> ⁻ <i>sm</i> ^r	dry	+

The complete lack of capability of these mutants to produce amylase was confirmed in various ways. These mutants did not show a halo of hydrolysis around colony, and starch in the agar just below the colonies was not hydrolysed even after two or three

day incubation. And amylase activity was not detectable in the culture media of the *amy*⁻ mutants by the assay method employed. When the mutants were cultivated in nutrient broth containing 0.2 % of soluble starch, the added starch remained unhydrolysed even after 72 hour cultivation, while in the case of the original *amy*⁺ strain, 1-168, a five hour cultivation was enough to hydrolyse the added starch completely. As can be seen in Fig. 1, when A2, A3 and A12 were grown in the minimal medium supplemented with DL-tryptophan and containing 0.2 % of starch as the sole carbon source, they did not show any substantial growth, though slight growth was observed during the initial twenty hours. The slight growth observed might be due to glucose contaminated in starch, since starch added was found to remain unhydrolysed. 1-168 showed slow but substantial growth after a longer lag period in the same medium. These results indicate that the isolated mutants lost the capability to produce active amylase into the culture medium.

Fig. 1. Growth of 1-168, A2, A3 and A12 in the minimal medium containing 0.2 % of starch as the sole carbon source.

---: on glucose, —: on starch. —●—: 1-168, —▲—: A2 —×—: A3, —■—: A12.

As shown in Table 1, all amylase negative mutants have the same nutrient requirement and drug sensitivity as the original strains. However, A3 and A11 formed a small, smooth, soft and moist colony in contrast to the rough and dry colony of the original strains. A3 and A9 were found to be unable to sporulate. Since it was found that transformation frequency of most of the *amy*⁻ mutants was low, clones of high transformability isolated by replica methods were used in the following genetic analyses.

TRANSFORMATION OF AMYLASE PRODUCING ABILITY. Transformation of amylase producing ability was attempted using the isolated *amy*⁻ mutants as recipients. It was impossible to select *amy*⁺ on the minimal agar containing starch as the sole carbon source. Thus, a competent cell culture of A8 exposed to DNA of 1-18 was plated on the nutrient agar containing 0.2 % starch. After colonies developed, a 0.1 N iodine solution was overflowed on the agar plate. By this method, amylase producing transformant could be detected as colony surrounded by halo of hydrolysis among halo-less colonies. As shown in Table 2, amylase producing colonies were found to occur

Table 2. Transformation of A12 to *amy*⁺ by DNA of 1-18

Experiment	Treatment*	Selection**	Colonies tested	<i>amy</i> ⁺ (%)
1	non	unselected	35175	35 (0.1)
		<i>try</i> ₂ ⁺ (2.7×10^{-4})	1815	81 (4.4)
	DNase	unselected	28080	0 (0)
		<i>try</i> ₂ ⁺ (0)	—	—
	control	unselected	13756	0 (0)
		<i>try</i> ₂ ⁺ (0)	—	—
2	non	<i>try</i> ₂ ⁺ (2.5×10^{-4})	223	7 (3.2)
	RNase	<i>try</i> ₂ ⁺ (2.3×10^{-4})	223	6 (2.7)

* 50 μ g/ml of DNase or RNase was added to the recipient cell suspension before exposing to the donor DNA. Control: the recipient cells were not exposed to the donor DNA.

** The recipient cells exposed to the donor DNA were plated on nutrient agar (unselected) and minimal agar (*try*₂⁺), containing 0.2 % of starch, respectively. In parenthesis, are shown the frequency of *try*₂⁺ per the recipient cells.

at the frequency of 1.0×10^{-3} . This frequency is comparable to that of *try*₂⁺ transformants, that is 2.7×10^{-4} . The occurrence of *amy*⁺ transformants was completely inhibited when 50 μ g/ml of DNase was present in the medium. This indicates that the amylase producers were elicited by DNA transformation. As can be seen in Table 2, it was also found that three to four percents of *try*₂⁺ transformants were producing amylase. The co-transfer index (Nester *et al.* 1963) of *try*₂ and *amy* was calculated to be as low as about 0.01, and this is too small to consider them as linked loci. But, since no selective marker linked to the *amy*⁻ locus was available to date, the transformation of amylase producing ability was followed by scoring *amy*⁺ colonies among *try*₂⁺ in the following experiment.

Table 3. Transformation of *amy*⁻ mutants to *amy*⁺ by DNA of 1-18

Recipient	<i>try</i> ₂ ⁺ transformants/ml	<i>try</i> ₂ ⁺ tested	<i>amy</i> ⁺ (%)
A2	1.5×10^5	306	27 (8.8)
A3	4.8×10^5	439	30 (6.8)
A6	3.4×10^5	672	64 (9.5)
A7	2.0×10^5	399	30 (7.5)
A8	8.3×10^3	883	59 (6.7)
A9	5.5×10^4	958	0 (0)
A11	7.0×10^4	526	0 (0)
A12	1.3×10^6	687	47 (6.7)

Table 3 shows experimental results of transformation of A2, A3, A6, A7, A8, A9, A11 and A12 by DNA of 1-18. Amylase producing transformants occurred among *try*₂⁺ transformants of A2, A3, A6, A7, A8 and A12 at the frequencies of 6 to 10 %. It should be noted that amylase producers were not found in the *try*₂⁺ transformants of A9 and A11. When more *try*₂⁺ transformants of A11 were examined, amylase producers were

found to occur at the frequency of approximately 0.1 % or less.

Recently, an *aro*⁻ marker was found to be linked to the *amy* gene (Yuki *et al.* 1967), and fine mapping of *amy*⁻ mutation sites, including A9 and A11, is now under way and the data will be reported in a separate paper.

ELECTROPHORESIS OF AMYLASE. Electrophoretic analyses of amylase produced by genealogically different strains of *B. subtilis* were performed by agar gel electrophoresis. Each strain, except the 1088 strain, showed only one amylase band which migrated to the cathode at pH 5.8. In 1088, three amylase bands were detected side by side. Among these three, the band located at the middle position showed the highest intensity. When a concentrated amylase sample of 1088 was electrophoresed, two or more faint bands appeared on both sides of the original three bands. The rate of migration of amylase was different from strain to strain, a total of six amylase types have so far been found in addition to the three isozymes of 1088 (Table 4).

Table 4. Types of amylase produced by various strains of *Bacillus subtilis*

Type of amylase	Band of amylase	Strain
	- 0 +	
1		44, 46
2		2-23, 38
3		189
4		1-168, 31, 45, 90, 149
5		37, 131, 158, 195
6		183, 186
1088		1088

Green *et al.* (1964) reported that a concentrated sample of amylase of SB1, a derivative of 1-168, showed two small bands in addition to one large band in starch gel electrophoresis. An amylase sample of the 1088 strain was reported by Green *et al.* to have two amylase bands; one was a large band moved to the anodal side at pH 8.4 and the other was a nonmigrating small band. The three bands detected in the present study seem to correspond to the large band observed by Green *et al.* (1964).

If a concentrated amylase sample of each strain had been run in the electrophoretic study, we might have detected additional faint amylase bands besides the sharp band really observed. Such amylase bands, if any, were, however, disregarded in the present study.

The six types of amylase were designated as amylase-1, 2, 3, 4, 5 and 6 according to their position in order from the original line to the cathode, *i. e.*, from right to left in Table 4. The amylase of the 1088 strain was designated as amylase-1088 since the relation of the three bands to the other amylases was not clear. The genetic symbols *amy*¹ through *amy*⁶ and *amy*¹⁰⁸⁸ were used for the genes which specify the types of these amylases of *B. subtilis*.

GENETIC ANALYSES ON AMYLASES WITH DIFFERENT ELECTROPHORETIC MOBILITY. Since DNAs of all the strains studied electrophoretically can transform 1-168 to prototroph, it was examined whether DNAs of the strains producing electrophoretically different types of amylase from that of 1-168 could transform the *amy*⁻ mutants to amylase producers, and what types of amylase the *amy*⁺ transformants produced. For this purpose, 2-23 (*amy*²⁺ *thr*⁻), 44 (*amy*¹⁺) and 1088 (*amy*¹⁰⁸⁸⁺) strains were employed as donors, which produced amylase-2, amylase-1 and amylase-1088, respectively. These types of amylase can readily be distinguished from amylase-4 produced by 1-168 in agar gel electrophoresis. Table 5 shows that all *amy*⁻ mutants used as recipients, except A11, can be transformed to *amy*⁺ by these DNAs.

Table 5. Transformation of *amy*⁻ mutants to *amy*⁺ by DNAs of 2-23, 44 and 1088 strains

Donor	Recipient	<i>try</i> ₂ ⁺ transformants/ml	<i>amy</i> ⁺ / <i>try</i> ₂ ⁺ (%)
2-23	A2	5.7×10 ³	4/235 (1.7)
	A3	2.1×10 ³	20/825 (2.4)
	A6	2 ×10 ⁴	3/177 (1.7)
	A11	4 ×10 ⁴	0/180 (0)
44	A2	4.5×10 ²	4/374 (1.1)
	A3	3.8×10 ³	13/857 (1.5)
	A6	8.5×10 ³	5/391 (1.3)
	A11	1.9×10 ³	0/295 (0)
1088	A2	7.9×10 ⁴	33/236 (14.0)
	A3	5.7×10 ⁴	23/228 (10.9)
	A6	2.1×10 ⁵	13/414 (3.1)

To examine the amylase types of these *amy*⁺ transformants by agar gel electrophoresis, amylase producing colonies of *try*₂⁺ transformants were purified and cultivated in nutrient broth with shaking for about 24 hours at 37°C, and the supernatant was electrophoresed as an enzyme sample. As a control experiment, amylase of *amy*⁺ transformants of A8 elicited by DNA of 1-18 as the donor was electrophoresed. As the result, all of twenty *amy*⁺ transformants examined produced the amylase-4, but no transformant producing an amylase with different mobility from that of amylase-4 was detected. On the contrary, among the *amy*⁺ transformants developed by DNA of 2-23, two types of amylase producers were found; one was the amylase-2 producer (*amy*²⁺) and the other the amylase-4 producer (*amy*⁴⁺). Some transformants were found to produce amylases which moved slightly faster or slower than amylase-2 or amylase-4, but these were included with amylase-2 or amylase-4 producers. No transformant was found which produces both types of amylase. As can be seen in Table 6, the frequency of amylase-4 producers appears to differ from recipient to recipient, but this data is not reliable since the total number of transformants examined is too small. DNA of 2-7, an *amy*⁻ mutant of 2-23, was also found to transform A8 to *amy*⁺ at a lower frequency. Among ten *amy*⁺ transformants obtained from this experiment, four produced the amylase-4 and the remaining six produced the amylase-2.

Table 6. Types of amylase produced by *amy*⁺ transformants elicited by DNAs of 2-23, 44 and 1088 strains

Donor	Recipient	Type of amylase		Total
		<i>amylase-2</i>	<i>amylase-4</i>	
2-23	A2	1	3	4
	A3	8	0	8
	A4	2	0	2
	A6	3	0	3
	A8	3	2	5
	A12	5	2	7
44		<i>amylase-1</i>	<i>amylase-4</i>	
	A2	7	2	9
	A3	9	1	10
	A6	8	2	10
	A7	11	4	15
	A8	5	2	7
1088	A12	7	0	7
		<i>amylase-1088</i>	<i>amylase-4</i>	
	A2	14	4	18
	A3	8	0	8
	A6	13	0	13
	A7	12	2	14
	A8	13	2	15
	A12	20	0	20

Similar results were obtained when the 44 and 1088 strains were used as donors (Table 6). Since the amylase of the 1088 strain was separable into three bands, it is of much interest to know if the *amy*⁺ transformants elicited by DNA of this strain show electrophoretically similar patterns to their donor or not. Unfortunately, a conclusive answer for this problem could not be drawn from the present experimental results. In many cases, the activity of amylase of the transformants was so low that only one band equivalent to the middle band of the donor was detected, but two other bands were hardly observable. No transformant having only side bands without the major band, corresponding to the original middle band, however, was found. So, it is not probable that the three isozymes of the 1088 strain are specified by the separate genes.

Since Green *et al.* (1964) concluded that amylase gene of 1088 was not allelic to that of SB1 and was integrated into the different locus without replacing the resident amylase gene, transformation of A8 to *amy*⁺ by DNAs of amylase-4 producing transformants

Table 7. Types of amylase produced by *amy*⁺ transformants elicited by DNAs of *amy*⁺ transformants

Donor*	Recipient	Type of amylase	
		<i>amylase-4</i>	<i>amylase-2</i>
amylase-2 producer (2-23)	A8	2	16
amylase-4 producer (2-23)	A8	29	0
amylase-4 producer (44)	A8	<i>amylase-4</i>	<i>amylase-1</i>
		30	0
amylase-4 producer (1088)	A8	<i>amylase-4</i>	<i>amylase-1088</i>
		21	0

* In parenthesis, are shown donor strains used in the first transformation.

were performed, to examine if the transformants carried the donor gene, *amy*², *amy*¹ and *amy*¹⁰⁸⁸. As shown in Table 7, amylase-2 producing transformant could transform the recipient to amylase-4 producers, as well as amylase-2 producers. On the contrary, amylase-4 producer could transform the recipient to only amylase-4 producers. This results seem to indicate that the amylase-4 producing transformants do not carry the donor gene such as *amy*², *amy*¹ and *amy*¹⁰⁸⁸, in spite of the fact that the transformants were elicited by these as donor.

DISCUSSION

Experimental results revealed that multi-molecular forms of amylase proteins were produced by *B. subtilis* strains and that DNAs from 2-23 (*amy*²⁺), 44 (*amy*¹⁺) and 1088 (*amy*¹⁰⁸⁸⁺) could transform the *amy*⁻ mutants isolated from 1-168 (*amy*⁴⁺) to amylase-4 producers, as well as to the donor type amylase producers. This seems to mean that *amy*⁴ is allelic to *amy*², *amy*¹ and *amy*¹⁰⁸⁸ genes and a site, named E site, which specifies the molecular forms of each amylase protein exist in these genes. The E site seems to be separate from, but closely linked to the mutation site, and the E site (*E*₄) which specifies the molecular form of amylase-4 seems to be retained in the *amy*⁴⁻ mutants in spite of the fact that these mutants do not have the capability to produce active amylase-4. Each of *amy*², *amy*¹ and *amy*¹⁰⁸⁸ genes may have also its own E site (*E*₂, *E*₁ and *E*₁₀₈₈) which specifies the molecular form of each of amylase-2, amylase-1 and amylase-1088. If recombination were to take place between the mutation site and the E site, the resultant *amy*⁺ transformants would produce amylase-4.

Green *et al.* (1964) studied a 1088 type amylase producing transformant of SB1, a derivative of 1-168, elicited by DNA of the 1088 strain. They concluded that the amylase gene of 1088 is not allelic to that of SB1 and is integrated into the different locus without replacing the resident amylase gene. They assumed that the amylase gene of 1088 repressed the expression of the recipient gene by some mechanism to explain the observation that the transformant produced only donor type amylase. However, this model is impossible to elucidate the evidence that *amy*⁴⁻ can be transformed to amylase-4 producers by *amy*²⁺, *amy*¹⁺ and *amy*¹⁰⁸⁸⁺ DNAs. The fact that, by DNA of 2-7, an *amy*⁻ mutants of 2-23, A8 was transformed to amylase-4 producers and amylase-2 producers is impossible to be explained, if *amy*⁴ and *amy*² are not allelic. The results shown in Table 7 seem also not to support their model.

Recently, multi-molecular forms (isozymes) of various enzymes have been reported in many organisms. With regard to amylase, Kikkawa (1963, 1964 and 1965) detected several isozymes of amylase in several strains of *Drosophila melanogaster*, *D. simulans* and *D. virilis*, and Ogita *et al.* (1963) reported more than seven amylase isozymes in adult house flies, *Musca domestica*. They proved that the amylase isozymes were genetically controlled by co-dominant allelic genes. On penicillinase of *Bacillus licheniformis*, which is an exo-cellular enzyme, Dubnau *et al.* (1965) studied transformation of penicillinase negative mutants to penicillinase producers by DNA of a strain which produced enzymatically and electrophoretically different penicillinase from that of the recipient strain, and they concluded that the two structure genes were strictly allelic.

If difference of electrophoretic mobility of proteins is attributable to difference of amino acid compositions, the amino acid sequences of these amylase proteins differ from each other at E site. Thus, the *amy* gene concerned with in this study is assumed to be the structure gene of amylase, in which E site is located, although no direct data showing that the *amy*⁻ was due to the mutation of the structure gene was obtained.

SUMMARY

Twelve amylase negative mutants were isolated from *Bacillus subtilis* 168 and its derivatives by irradiating spores with ultraviolet light. Using these mutants as recipients, genetic analyses of gene controlling the production of amylase in *B. subtilis* were performed by DNA transformation. Agar gel electrophoretic study showed that genealogically different strains carried different amylase genes and resulted in the production of amylases with different electrophoretic mobility. Data obtained indicate that these amylase genes are allelic to each other and a site named E within the structure gene specifies the molecular forms of each amylase protein.

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