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Transformation of *Bacillus subtilis* in α-Amylase Productivity by Deoxyribonucleic Acid from *B. subtilis* var. *amylosacchariticus*

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Deoxyribonucleic acid (DNA) of *Bacillus subtilis* var. *amylosacchariticus* showed almost the same ability as *B. subtilis* Marburg to induce transfer of several genetic markers in DNA-mediated transformation. DNA-DNA hybridization data also showed an intimate relationship between the two strains. Genetic elements involved in the production of extracellular α-amylase (EC 3.2.1.1.) in *B. subtilis* var. *amylosacchariticus* were studied by using DNA-mediated transformation. Two Marburg derivatives, NA20(*amyR2*) and NA20-22(*amyR1*), produced about 50 and 10 U of α-amylase per mg of cells, respectively, whereas *B. subtilis* var. *amylosacchariticus* produced as much as 150 U of the enzyme per mg of cells. When *B. subtilis* var. *amylosacchariticus* was crossed with strain NA20-22 as recipient, transformants that acquired high α-amylase productivity (about 50 U/mg of cells) were obtained. Genetic analysis revealed that a regulator gene (*amyR*) for α-amylase synthesis was found in *B. subtilis* var. *amylosacchariticus*, as in the case of *B. natto* 1212 (*amyR2*) and *B. subtilis* Marburg (*amyR1*). The allele was designated *amyR3;* it is phenotypically indistinguishable from *amyR2*, but is readily distinguishable from *amyR1*. The presence of *amyR3* was not sufficient for an organism to render production of an exceptional amount of α-amylase. Extra-high α-amylase producers could be obtained by crossing *B. subtilis* var. *amylosacchariticus* as donor with strain NA20 as recipient. The transformants produced the same or even greater amounts of the enzyme than the donor strain. Results suggest the presence of another gene that is involved in the production of the exceptional amount of α-amylase.

In our previous papers (14, 15), a specific regulator gene was reported to be involved in α-amylase (EC 3.2.1.1.) production in *Bacillus subtilis*. Gene *amyR* was shown to regulate the rate of α-amylase synthesis in this organism; it is closely linked to the structural gene (*amyE*) of the enzyme (14, 15, 18, 19). The gene could be transferred from *B. natto* 1212 to *B. subtilis* Marburg by deoxyribonucleic acid (DNA)-mediated transformation (14). *B. natto* 1212 carries *amyR2* and produces about 50 U of α-amylase per mg of cells, whereas *B. subtilis* Marburg bears *amyR1* and produces about 10 U of the enzyme per mg of cells. A highly amylolytic bacillus was isolated from soil by Fukumoto (2). The strain, *B. subtilis* var. *amylosacchariticus*, produces as much as 150 U of α-amylase per mg of cells. The α-amylases produced by these three strains fall into the category containing the so-called bacterial saccharifying-type α-amylase and show similar immunological properties (3; Matsuzaki et al., Biochim. Biophys. Acta, in press).

To investigate the regulatory mechanisms of such exceptional productivity of α-amylase in *B. subtilis* var. *amylosacchariticus*, we attempted to analyze genetic elements involved in the production of the enzyme by DNA-mediated transformation. Data show that several genetic markers including *amyR* and *amyE* could be transferred to *B. subtilis* Marburg by DNA from *B. subtilis* var. *amylosacchariticus* at the same frequencies as for homologous crosses. Data are also presented that infer the involvement of genetic elements other than *amyR* in the "extra-high" productivity of α-amylase in this organism.

MATERIALS AND METHODS

Organisms. *B. subtilis* strains 6160 (*metB5 purB6 trp-160 amyR1 amyEm*$^+$) and Mu8u5u5 (*leu met thr*)

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are derivatives of strain 168 (1). *B. subtilis* W23 (prototroph) and *B. natto* 1212 (*amyR2 amyEn*$^+$) were donated by Y. Ikeda of this Institute. *B. subtilis* var. *amylosacchariticus*(prototroph) was a gift from J. Fukumoto of Osaka City University (2, 3). This organism will hereafter be referred to as *B. subtilis* SAC. Phenotypic and genotypic characters that pertain to the production of α-amylase and proteases in *B. subtilis* 6160, NA20, and NA20-22, *B. natto* 1212, *B. subtilis* SAC, and *B. subtilis* YY88 are presented in Table 1. The properties of *B. subtilis* YY88 were discussed previously (17).

Strains SAC-2 and 6160-1 were streptomycin-resistant (Str^R) derivatives of strains SAC and 6160, respectively. They were obtained by treating the parental strains with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Strain 6160-2 was an *aro-116* derivative of strain 6160-1. Strains NA20 (*metB5 purB6 amyR2 amyEn*$^+$) and NA64 (*metB5 purB6 amyR2 amyEm*$^+$) were obtained by a cross of *B. natto* 1212 with strain 6160 as recipient (14, 15). Strain NA20-22 was an *amyR1* derivative of strain NA20, and strain NA6408 was an *aro-116* derivative of strain NA64. Strain SA11 (*metB5 purB6 trp-160 amyR3 amyEs*$^+$) was a transformant that acquired high α-amylase productivity and streptomycin resistance in a cross of *B. subtilis* SAC-2 with strain 6160 as recipient. Strain SA11-6 was an *aro-116* derivative of strain SA11.

Media and culture conditions. The composition of bouillon-yeast extract medium and of minimal medium were reported previously (15). Cells were grown in bouillon-yeast extract medium with shaking at 30 or 37 C as indicated.

Assay of enzyme activity. Determination of α-amylase activity was described previously (15). One unit of enzyme activity was defined as hydrolysis of 100 μg of soluble starch in 1 min at 40 C. α-Amylase productivity was determined by measuring the halo around a colony grown on a bouillon-yeast extract agar plate containing 1% soluble starch, after it was sprayed with 0.01 M KI-I_2 solution. Protease activity was assayed by a modified method of Hagihara (5, 11). Protease productivity was determined by measuring the halo around a colony grown on a bouillon-yeast extract agar plate containing 1% casein (11).

Determination of α-amylase type. The α-amylase type was deduced from thermostability and electrophoretic mobility of the enzyme produced by a given strain. Thermostability was measured as follows. α-Amylase solution was diluted with 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.6) containing 0.001 M calcium acetate to give about 5 U of the enzyme, and was treated at 65 C for 5 min. The mixture was quickly chilled in an ice bath, and the remaining enzyme activity was measured. S-type and M-type α-amylases (produced by *B. subtilis* SAC and *B. subtilis* Marburg, respectively) were inactivated less than 50% after the heat treatment, whereas N-type α-amylase (produced by *B. natto* 1212) was inactivated more than 90%. S-type and M-type α-amylases were characterized by polyacrylamide gel electrophoresis (Matsuzaki et al., in press).

Procedure for transformation. Transforming DNA was prepared from exponentially growing cells by the method of Saito and Miura (9), and transformation experiments were performed by the method of Saito et al. (8). Co-transfer index (CI) between *amyR* and *amyE* (or *amyR* and *aro-116*) was calculated by the method of Nester and Lederberg (7). To use a general notation, in cross $a^+b^+ \times a^-b^-$, giving transformant types a^+b^+, a^+b^-, and a^-b^+, and assuming $a^-b^+ \simeq a^+b^-$, the CI = $a^+b^+/(a^+b^+ + 2\ a^+b^-)$. For example in the reciprocal transfer mentioned in Results, CI (between *amyR1* and *amyE*) = *amyR1-amyEn*/(*amyR1amyEn* + 2 *amyR1amyEs*), and CI (between *amyR* and *aro-116*) = *aro*$^+$*amyR1*/(*aro*$^+$ *amyR1* + 2 *aro*$^+$*amyR3*).

TABLE 1. *Phenotypes and relevant genotypes of strains used in relation to the production of α-amylase and protease*

Strains	Phenotype			Relevant genotype of *amy*[a]
	Productivity of α-amylase[b]	Type of α-amylase[c]	Productivity of protease[d]	
B. subtilis 6160	Amy$^+$	M	Pro$^+$	*amyR1 amyEm*
B. subtilis NA20	Amy-sup	N	Pro$^+$	*amyR2 amyEn*
B. subtilis NA20-22	Amy$^+$	N	Pro$^+$	*amyR1 amyEn*
B. natto 1212	Amy-sup	N	Pro-sup	*amyR2 amyEn*
B. subtilis SAC-2	Amy-extra	S	Pro-sup	*amyR3 amyEs Amy-extra*[e]
B. subtilis YY-88	Amy-extra	M	Pro-sup	*amyR2 amyEm pap*[f]

[a] The genetic symbols of *amyR* and *amyE* mean regulator and structural genes of α-amylase (15).

[b] α-Amylase levels of Amy$^+$, Amy-sup, and Amy-extra at the stationary phase of growth were about 10, 50, and 150 U/mg of cells, respectively.

[c] Determination of α-amylase type was described in the text.

[d] The strains produced three proteolytic enzymes when casein was used as substrate (11). A total of the three enzyme activities is presented. Proteolytic enzyme activity levels of Pro$^+$ and Pro-sup at the stationary phase of growth were about 5 and more than 20 U/ml of culture fluid, respectively.

[e] This strain should have a genetic element(s) related to the production of extra-high level of α-amylase.

[f] The genetic symbol *pap* designates production of α-amylase and protease (17).

Preparation of DNA for hybridization experiments. DNA was prepared by the method of Marmur (6). For further purification, crude DNA solution was digested by pancreatic ribonuclease A and fungal ribonuclease T_1, and treated twice with chloroform-isoamylalcohol. Tritiated DNA of *B. subtilis* 6160 was prepared as follows. The bacterium was grown in a minimal medium containing the necessary requirements and labeled with [^{3}H]thymidine (0.4 mCi) for 40 h at 30 C. The cells were harvested by centrifugation, and DNA was prepared as described above.

DNA-DNA hybridization. DNA membrane filters were prepared as described by Gillespie and Spiegelman (4). Heat-denatured DNA (10 μg) from each strain was loaded on membrane filters (HAWP, 0.5 by 1 cm; Millipore Corp.), dried overnight, and treated for 2 h at 80 C. Hybridization was carried out as described by Warnaar et al. (12). Various amounts of denatured [^{3}H]DNA from *B. subtilis* 6160 were added to test tubes containing the DNA filters and incubated at 60 C for 3 h. Filters were rinsed in 0.003 M Tris buffer (pH 9.4) to remove nonhybridized [^{3}H]DNA and dried. Radioactivity was measured by a liquid scintillation counter.

RESULTS

Genetic relationship between B. subtilis Marburg and B. subtilis SAC. Transfer frequencies of several genetic markers by DNAs of *B. subtilis* W23 and *B. subtilis* SAC strain are shown in Table 2. Three Marburg strains, 6160, Mu8u5u5, and NA6408, which possessed different nutritional requirements, were used as recipient strains. DNAs of the the two donor strains had almost the same ability to induce DNA-mediated transformation in any marker tested. The transformation data thus suggested that *B. subtilis* SAC is closely related to *B. subtilis* Marburg.

To obtain further information on the relationship of these bacilli, the degree of DNA-DNA hybrid formation was examined (Fig. 1). Ten micrograms of DNA from the strain to be tested was loaded on a filter and, when 0.4 μg of the tritiated DNA from strain 6160 was added, the degree of hybrid formation in the case of *B. subtilis* SAC was 85% that of homologous DNA. The degree of hybrid formation for DNAs from *B. natto* 1212 and *B. amyloliquefaciens* were 80 and 40%, respectively, in good agreement with observations by other investigators (10, 13). In the case of phage λ DNA it was less than 5%. These results indicate that the DNA of *B. subtilis* Marburg and that of *B. subtilis* SAC contains many homologous regions in the nucleotide sequences, and suggest the close taxo-

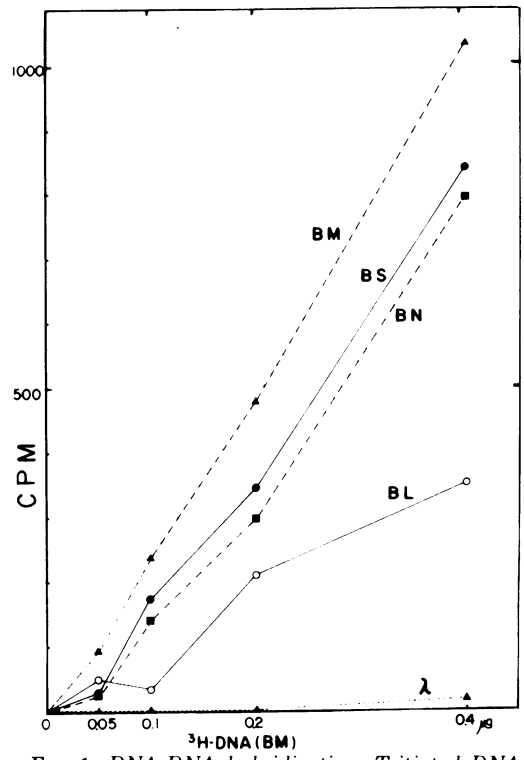

FIG. 1. *DNA-DNA hybridization. Tritiated DNA from B. subtilis 6160 and unlabeled DNAs from B. subtilis 6160 (BM, ▲), B. subtilis SAC (BS, ●), B. natto 1212 (BN, ■), B. amyloliquefaciens (BL, ○), and phage λ (λ, ▲) were prepared and incubated as described in the text.*

TABLE 2. *Transfer frequencies of several genetic markers*[a]

Donor	No. of transformants[b] (× 10^8)					
	Mu8u5u5[c]			6160[c]		NA6408[c]
	thr$^+$	*leu*$^+$	*met*$^+$	*ade*$^+$	*trp*$^+$	*aro*$^+$
B. subtilis W23	0.9	2.8	0.8	43	69	5.5
B. subtilis var. *amylosacchariticus*	1.5	1.2	1.4	75	88	21

[a] 13.2 μg of DNA per ml was used for each cross.

[b] Number of recipient cells per 0.1 ml was: Mu8u5u5, 0.8 × 10^7; 6160, 1.6 × 10^7; NA6408, 2.0 × 10^7. Numbers of transformants in the table were expressed per 10^8 recipient cells.

[c] Recipient.

nomical relationship between the two strains.

Transfer of α-amylase high productivity. *B. subtilis* NA20-22 (*amyR1 amyEn*$^+$) is an *amyR1* derivative of the N-type α-amylase producer. It was obtained from strain NA20 (*amyR2 amyEn*$^+$) by introduction of *amyR1* from strain 6160 (*amyR1 amyEm*$^+$) by DNA-mediated transformation. *B. subtilis* SAC-2 produced about 150 U of S-type α-amylase per mg of cells. Transformation of α-amylase high productivity into *B. subtilis* NA20-22 by DNA from strain SAC-2 was examined: 1,907 Str^R colonies were picked, 27 of which showed high α-amylase productivity; 25 were S type and 2 were N type; CI = 0.86. All of the transformants with a character of α-amylase high productivity produced 30 to 50 U of the enzyme per mg of cells (Table 3). These activity levels are similar to those produced by strains carrying *amyR2*, such as *B. subtilis* NA20 and *B. natto* 1212 (14), but are definitely lower than that of the donor strain (about 150 U/mg of cells). Thus, it is possible that *B. subtilis* SAC-2 bears an *amyR2*-like character, as does *B. natto* 1212, and that it carries, in addition, another gene(s) that is concerned with the production of the "extra-high" amount of α-amylase. The transformants obtained in this experiment gained only the *amyR2*-like character, but not the other gene(s), from strain SAC-2.

Since *amyR1* in *B. subtilis* Marburg and *amyR2* in *B. natto* 1212 are linked to marker *aro-116* (14, 16, 19), the linkage between *aro-116* and the *amyR2*-like character in strain SAC was investigated. In a cross of SAC-2 as donor with 6160-2 (*aro-116 amyR1 amyEm*$^+$), as recipient, of 180 *aro*$^+$ transformants, 89 were α-amylase high producers. The *amyR2*-like character was linked to *aro-116* with a CI of 0.33. The value of 0.33 was comparable to that (CI = 0.38) obtained in the cross of *B. natto* 1212 as a donor with *B. subtilis* 6160 as a recipient (14). Therefore, it is likely that *B. subtilis* SAC carries an *amyR* gene that is phenotypically very similar to *amyR2* of *B. natto* 1212. We designated the *amyR* allele of *B. subtilis* SAC as *amyR3*. Although one cannot distinguish *amyR3* from *amyR2* phenotypically, the two strains originated from natural sources; it is reasonable to assume that the markers may have differences in their nucleotide sequences. The type of α-amylase produced by the transformants was judged by thermostability of the enzymes. Out of 27 high α-amylase producers, 25 produced the donor-type α-amylase (S type) and 2 produced recipient type (N type). Accordingly, the CI of *amyR3* and *amyEs* (structural gene of S-type α-amylase) was calculated to be 0.86. In the present experiment we obtained three recombinant types: (*amyR3 amyEs*$^+$), (*amyR3 amyEn*$^+$), and (*amyR1 amyEn*$^+$).

TABLE 3. *Production of α-amylase in the parental and representative transformant strains*[a]

Strain		α-Amylase activity (U/mg cells)
Donor	SAC-2	156
Recipient	NA20-22	9.5
Transformant	SA11	52.5
	SA12	33.7
	SA13	38.3
	SA14	29.7
	SA15	45.2
	SA16	47.3
	SA18	31.2

[a] Cells were grown in BY medium at 37 C for 14 h.

Since *B. subtilis* SAC could not be a recipient in DNA-mediated transformation because of lack of competence, we constructed a Marburg strain that gained the α-amylase genes (*amyR3* and *amyEs*) from strain SAC-2. The strain also received *aro-116*, and the resulting strain, SA11-6(*purB6 trp-160 aro-116 amyR3 amyEs*$^+$), was used as a recipient in the reciprocal-cross experiments. Strain NA20-22 (*amyR1 amyEn*$^+$) was used as a donor. The CI of *aro-116* and *amyR1* was calculated to be 0.41. The type of α-amylases produced by 209 *amyR1* transformants (of 360 *aro*$^+$ transformants) was examined: 205 were found to produce donor-type α-amylase (N type) and 4 produced the recipient type (S type). The CI of *amyR1* and *amyEn* was accordingly calculated to be 0.96. In this cross we could obtain the last one of the four possible recombinant types: *amyR1 amyEs*$^+$. From the above data, the order of *amyR-amyE-aro-116* could be deduced, since among *aro*$^+$ transformants 57% was *amyR1-amyEn*, 42% were *amyR3-amyEn* plus *amyR3-amyEs*, and only 1% were *amyR1-amyEs*.

The results indicated, in accordance with the observations for *B. subtilis* Marburg and *B. natto* 1212 (14–16), that (i) *B. subtilis* SAC carries an *amyR* allele, designated *amyR3*, and was phenotypically indistinguishable from *amyR2* of *B. natto* 1212; (ii) the *amyR* gene was segregated from the α-amylase structural gene (*amyE*); (iii) the two genes are closely linked to each other, and both genes are linked to *aro-116;* and (iv) the order *amyR-amyE-aro-116* was confirmed (16, 18).

Transfer of extra-high productivity of α-amylase. Since in a cross of *B. subtilis* SAC-2 as a donor with strain NA20-22 (*amyR1 amyEn*$^+$) we obtained transformants that ac-

quired only *amyR3*, but we could not obtain any transformant that produced as much as 150 U of α-amylase per mg of cells, we suspected the presence of additional genes that corresponded to the production of the exceptional amount of α-amylase; we therefore tried another selection in which strain NA20 (*amyR2 amyEn*$^+$) was used as a recipient.

A mutant that had the genetic character causing an increase in the production of both α-amylase and protease simultaneously was isolated in our laboratory from *B. subtilis* Marburg by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment (17). This mutation seemed to have occurred at a single gene that was not linked to *aro-116*. The gene was named *pap* (productivity of amylase and protease). When *pap* and *amyR2* coexisted in one strain, for example *B. subtilis* YY88 (Table 1; 17), the two genes were expressed synergistically, and the strain produced as much as 150 U of α-amylase per mg of cells together with about 60 U of protease per ml. The presence of gene *pap* resulted in an increase of the three proteolytic enzymes (neutral and alkaline proteases and esterase) and of α-amylase (17). A gene (*nprR*) that specifically regulates the production of neutral protease in *B. subtilis* was revealed to be independent of *amyR* or *pap* (11). The results of detailed study on *pap* will be published elsewhere.

B. subtilis SAC has the ability to produce approximately the same levels of α-amylase and proteases as *B. subtilis* YY88, an *amyR2-pap*-bearing strain (Table 1). Therefore, we assumed that *B. subtilis* SAC might carry a *pap*-like gene. Strain SAC-2 (Str^R) was used as a donor at a high DNA concentration with recipient NA20 (*amyR2 amyEn*$^+$), and Str^R transformants were selected primarily. Among Str^R transformants, protease high producers were selected on bouillon-yeast extract agar plates containing 1% casein. Productivity of α-amylase of the protease high producers was then checked in a liquid medium, since it is difficult to distinguish the high α-amylase producers and the extra-high producers by measuring halo sizes on plates containing soluble starch. Nineteen protease high producers could be picked among 7,315 Str^R transformants, and 3 α-amylase extra-high producers were found among the 19 transformants. Figure 2 shows the time courses of growth and α-amylase production by two of three such transformants (NA20-S15 and NA20-S17), the donor strain (SAC-2) and the recipient strain (NA20). Growth curves of the four strains did not show much difference. Transformants NA20-S15 and NA20-S17 produced a comparable or greater amount of α-amylase than the donor strain. The reason why strain NA20-S17 produced almost twice as much α-amylase as the parent is now under investigation.

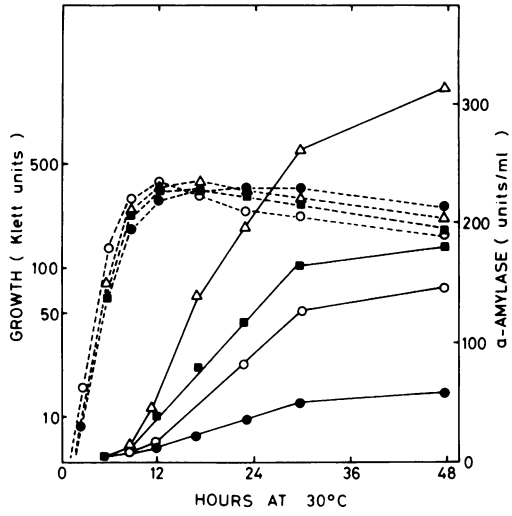

FIG. 2. *Growth curves and α-amylase production of parental strains and transformants. Cells were cultured in BY medium at 30 C. At times indicated, cell density (broken line) and α-amylase activity (solid line) in the culture fluids were measured. Symbols:* ○, *donor strain B. subtilis SAC-2;* ●, *recipient strain B. subtilis NA20;* ■, *transformant strain NA20-S15;* △, *transformant strain NA20-S17.*

It seems likely from the above data that characters for extra-high α-amylase production and high protease production may segregate as independent markers, since only 3 of 19 high-protease producers acquire the extra-high α-amylase character. It is noteworthy that the α-amylase produced by the transformants was of the N type and that no transformant was detected that simultaneously produced two types (S and N) of α-amylase.

DISCUSSION

Two strains of highly amylolytic bacilli were isolated from soil by Fukumoto (2). One produced a large amount of liquefying-type α-amylase and was classified as *B. subtilis* var. *amyloliquefaciens*. Welker and Campbell (13) later claimed that the strain had almost no relationship with *B. subtilis* Marburg strain according to DNA-DNA hybridization, transduction, and transformation data, and they proposed to call the strain *B. amyloliquefaciens* strain F. The other strain produces a large amount of saccharifying-type α-amylase and

was classified as *B. subtilis* var. *amylosaccharitícus* (3); the strain has been referred to as SAC strain in this report. Unlike *B. amyloliquefaciens*, *B. subtilis* SAC exhibited an intimate relationship with *B. subtilis* Marburg as far as DNA-DNA hybridization and transformation data were concerned (Table 2 and Fig. 1). Results of a comparative study of α-amylases produced by these bacilli (Matsuzaki et al., in press) are consistent with the above-mentioned genetic data.

Gene *amyR* has been reported to regulate the synthesis of α-amylase in *B. subtilis* (14, 15, 18). The gene could be expressed in two phenotypes, high and low production of α-amylase; *B. natto* 1212 carries *amyR2* and produces about 50 U of α-amylase per mg of cells, whereas *B. subtilis* Marburg carries *amyR1* and produces about 10 U/mg of cells (14). From the genetic analyses presented here, it was suggested that *B. subtilis* SAC carries *amyR3* in addition to other genetic element(s) that determine the production of as much as 150 U of α-amylase per mg of cells. A strain that acquired *amyR3* from strain SAC produces about the same amount of the enzyme as the *amyR2*-carrying strains. *amyR3* was closely linked to the structural gene of α-amylase (*amyEs*), with a CI of 0.86 or 0.96 (reciprocal cross) (see above). These values were similar to that of *amyR1* and *amyEm* (CI = 0.81) obtained in *B. subtilis* Marburg (14). The *amyR* gene in *B. subtilis* Marburg and *B. natto* 1212 has been known to be linked to *aro-116* with a CI of 0.41 and 0.38, respectively (14). In the case of *B. subtilis* SAC, the CI value of *amyR3* and *aro-116* was 0.33 or 0.41 (reciprocal cross) (see above); therefore, it is suggested that the gene structure around the α-amylase gene in strain SAC is similar to that of *B. subtilis* Marburg and *B. natto* 1212.

The transformants that acquired *amyR3* from *B. subtilis* SAC produced about 30 to 50 U of α-amylase per mg of cells (see above). The level of the activity was similar to that produced by *B. natto* 1212 or the *amyR2* transformants obtained by a cross of *B. natto* 1212 as a donor with strain 6160 (14). Since strain SAC produced as much as 150 U of the enzyme per mg of cells the acquisition of *amyR3* was not sufficient to cause production of an exceptional amount of α-amylase, and thus *B. subtilis* SAC must carry another genetic element(s) that is concerned with the production of an extra-high amount of the enzyme. As our data indicate, we can obtain such extra-high producers of α-amylase (see above and Fig. 2). The transformants obtained simultaneously acquired a character of high protease productivity. A gene involved in high productivity of protease in *B. subtilis* has been known to be independent of the *amyR* gene (11, 15). On the other hand, a mutant of *B. subtilis* Marburg was isolated in our laboratory that simultaneously produced large amounts of both α-amylase and protease by a single gene mutation. Gene *pap* was proposed for the genetic element associated with this phenomenon (19). A strain that acquired *amyR2* and *pap* produced about 150 U of α-amylase per mg of cells (17). From the above observations, it seems likely that *B. subtilis* SAC carries genetic elements corresponding to the production of extra-high α-amylase and high protease as independent markers. Another possibility is that *B. subtilis* SAC, although it was isolated from soil, had gained the *pap*-like gene in addition to *amyR3* by natural mutational events and that α-amylase extra-high-producing transformants acquired this gene.

Since one of the extra-high α-amylase-producing transformants obtained here produced as much as 300 U of the enzyme per mg of cells, and we cannot explain fully the observation right now, it will require more detailed studies to understand the overall mechanisms of α-amylase production in *B. subtilis*. Up to now, no transformant could be detected that produced two types of α-amylase simultaneously, and thus it seemed unlikely that the extra-high α-amylase producer has two or more α-amylase structural genes. Further genetic studies are in progress to elucidate the mechanism of such an extra-high productivity of α-amylase.

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