MEMBRANE MUTATION RELATED TO THE PRODUCTION OF EXTRACELLULAR α-AMYLASE AND PROTEASE IN BACILLUS SUBTILIS

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Mutants which had a genetic character to increase the production of both α-amylase and protease simultaneously, were isolated from a transformable strain of Bacillus subtilis Marburg by NTG treatment. This mutation seems to have occurred at a single gene of the bacterial chromosome and was not linked to aro_{116} which was closely linked to the α-amylase gene. When this mutation and an α-amylase regulator gene ($amyR^h$) coexisted in one strain, their synergistic effect on extracellular α-amylase production was observed. The introduction of this mutation resulted in a loss of competence for the transformation. The SDS disc gel electrophoretic profiles of the membrane proteins from the original strain, the mutants and transformants with this mutation showed a remarkable difference in one component.

Participation of a regulator gene (amyR) in α-amylase production has been confirmed in B. subtilis Marburg strain (1, 2). When $amyR^h$ was transferred from B. natto IAM 1212 (high α-amylase productivity, about 40 units/ml of culture medium) to B. subtilis (low α-amylase productivity, about 10 units/ml), the transformants produced about 40 units/ml of α-amylase. The amyR was closely linked to the α-amylase structure gene and was also linked to aro_{116}. The order of these genes was confirmed to be amyR - amylase structure gene - aro_{116} (1, 2).

In order to isolate mutants of amyR, a transformable strain of B. subtilis Marburg was treated with NTG. Among the mutants obtained with high α-amylase productivity, 6 strains produced higher amount of extracellular protease simultaneously but not ribonuclease. The stimulation of the production of the two enzymes was found to be controlled by an another gene which was not linked to aro_{116}. The gene was tentatively named pap.

In this paper, some characteristics of pap and a synergistic effect of $amyR^h$ and pap on the production of α-amylase will be described.

MATERIALS AND METHODS

B. subtilis Marburg strain, 6160 (ade, met, try, leu), 6160-1 (ade, met,

try, leu, str-r), 6160-2 (aro_{116}, met, try, leu, str-r), B. natto IAM 1212 (a stock culture at Institute of Applied Microbiology, The University of Tokyo) were used. The production of α-amylase and protease was not changed in B. subtilis Marburg 6160, 6160-1 and 6160-2. Other strains in text were obtained by transformation or by N-methyl-N'-nitro-N-nitrosoguanidine treatment (NTG, 100 μg/ml for 20 min). DNAs used in transformation were isolated according to the method of Saito and Miura (3). The procedures employed in transformation experiments were those described by H. Yoshikawa (4). Amylase activity was assayed by modified blue value method of Fuwa (5). Protease activity was assayed by the casein digestion method of Hagihara (6). Amylase or protease activity on plate was measured by size of halos around colonies on 1 % soluble starch- or 1 % casein-agar plate. Membrane preparations were obtained according to modified method of Mizushima et al. (7) and were treated in a mixture of 1 % SDS and 1 % β-mercaptoethanol at 70° C for 20 min, and layered on the polyacrylamide disc gels (0.6 x 16 cm). Electrophoresis was performed under constant current of 2 mA per gel for about 18 hrs.

RESULTS AND DISCUSSION

Fifteen strains with high α-amylase productivity were isolated from 115,000 colonies of B. subtilis Marburg 6160-1 and 6160-2 which had been treated by NTG. Six strains of them produced an increased amount of protease simultaneously and were named "AP-mutant". After 34 hours culture, the parental strain produced 11.4 units/ml of α-amylase and 5.5 units/ml of protease, whereas, YN-9, one of these "AP-mutants", elaborated 17.1 units/ml of α-amylase and 41.3 units/ml of protease (Table). Their growth curves showed that the high production of α-amylase and protease in the mutants were not due to an increase in total cell number but due to increased rates of α-amylase and protease production. This view is also supported by the fact that the productivity of extracellular ribonuclease was not affected by this mutation. When DNAs of the mutants were applied to a normal transformable strain, all the transformants (67/67) select-

Table: Effect of $amyR^h$ and pap on the production of α-amylase and protease

Strains	Possible regulator gene	Enzyme activity α-amylase (Units/ml)	Enzyme activity protease (Units/ml)	Origin
B. subtilis Marburg 6160	$amyR^1$, pap^+	11.4	5.5	Parental strain
YN-9	$amyR^1$, pap^-_9	17.1	41.3	NTG* treatment
YN-118	$amyR^1$, pap^-_{118}	22.3	34.5	NTG* treatment
B. natto IAM 1212	$amyR^h$, pap^+	42.8	81.0	Stock culture of IAM
NA-64	$amyR^h$, pap^+	46.0	4.0	Transformation of 6160 by B.natto DNA
YY-88	$amyR^h$, pap^-_9	143.0	64.0	Transformation of NA-64 by YN-9 DNA
YY-110	$amyR^h$, pap^-_{118}	140.0	57.7	Transformation of NA-64 by YN-118 DNA

Each strain was cultured in BY-medium (10) at 30° C for 34 hrs, cells were removed by centrifugation and then amylase and protease activities in the supernatants were measured by a modification of blue value method of Fuwa (5) and by the casein digestion method of Hagihara (6) respectively.

*) NTG: N-methyl-N'-nitro-N-nitrosoguanidine.

ed by high protease productivity also showed high α-amylase productivity. This fact strongly suggests that this mutation occurred at a single gene. Therefore, this kind of mutation was designated tentatively as "pap" (productivity of α-amylase and protease). But we did not determine yet whether this mutation was a single event or not, because the frequency of natural reversion of these mutants could not be measured easily.

Yamaguchi et al. (2) clarified that the amyR regulated the production of α-amylase and that it was linked to both an α-amylase structure gene and aro_{116}. The linkage of pap and amyR to aro_{116} was compared by means of transformation of Marburg strain (6160-2, aro_{116}) using DNA from YN-9 with pap, and from B. natto with $amyR^h$. The co-transfer index (C.I.) of aro_{116} and high α-amylase productivity was 0.01 for YN-9 and 0.32 for B. natto. These C.I.s show that

pap in YN-9 is not linked to aro_{116}, while $amyR^h$ in *B. natto* does as reported earlier (2). Therefore, we concluded that *amyR* and *pap* are different.

To elucidate the effects of coexistence of *pap* and $amyR^h$ in a cell, DNA from YN-9 was transferred to *B. subtilis* NA-64, which had gained $amyR^h$ from *B. natto* by a previous transformation. As shown in the Table, transformants were isolated (YY-88 strain is a representative) which produced about 140 units/ml of α-amylase. Protease production was also 12 to 16 times that of the recipient strain. A similar experiment to introduce *pap* into NA-64 ($amyR^h$) was performed using DNA from YN-118, another "AP-mutant" strain. Almost the same results were obtained; the YY-110 strain is illustrated in the Table.

Transformants with high protease productivity were isolated by the transformation of the Marburg strain with DNA from *B. natto* (H. Uehara *et al.* unpublished data). Introduction of *pap* into the transformants resulted in the further increased production of protease (70 times that of the original Marburg strain), as in the case of α-amylase.

These synergistic effects of the two genes suggest that at least two kinds of genes regulate the amount of extracellular α-amylase and protease production in *B. subtilis*. One of them is a specific regulator gene for each enzyme, like *amyR*, while the other is a common regulator gene for two or more kinds of enzymes, like *pap*. The existence of the latter kind of regulator gene was also reported in *Neurospora crassa*, in which the production of some extracellular enzymes were simultaneously stimulated by one gene mutation (8). The function of these gene may be related to some common regulation sites for the production of extracellular enzyme such as secretion site of them. It should be noted that *pap* did not show any effects on the production of extracellular ribonuclease.

During the experiment in the effects of coexistence of *pap* and $amyR^h$ in one strain, we realized that all attempts to transform the strains with *pap* mutation were unsuccessful. The abilities of competence in *pap* mutants were reinvestigated concerning to methionine and adenine requirement. The result

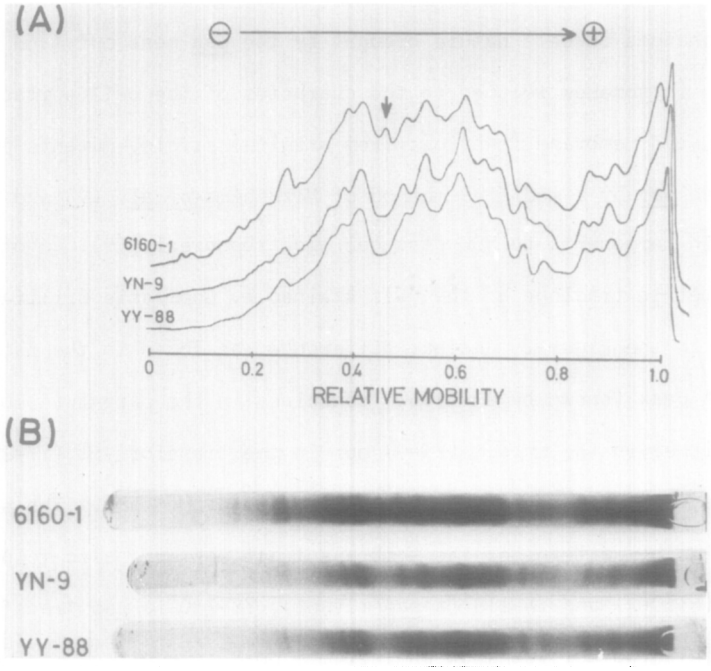

Figure: Denstmetric tracings (A) and photographs (B) of SDS polyacrylamide disc gels of membrane preparations. Membrane preparations were obtained according to a modified method of Mizushima et al. (7). Cells were cultured in BY-medium for 8 hrs at 30° C, harvested, washed once with 5 mM TrisHCl, pH 7.0 containing 0.85 % NaCl, and treated by lysozyme in the presence of 0.5 M sucrose. The resulted protoplasts were disrupted in 5 mM phosphate buffer, pH 7.0 containing 5 mM $MgCl_2$ and 50 μg/ml DNase and were homogenized. The precipitated of the homogenates by a centrifugation (15,000 x g, 20 min) were further treated with pH 8.0 without Mg^{++} in order to remove ribosomes. After the suspensions were centrifuged at 15,000 x g for 20 min and the pellets were washed under the same conditions, the suspensions of them were centrifuged at 7,500 x g for 15 min. The membrane preparations were precipitated from the supernatants by a centrifugation (60,000 x g, 60 min). The membrane preparations were treated in a mixture of 1 % SDS and 1 % β-mercaptoethanol at 70° C for 20 min and layered on the polyacrylamide disc gels (0.6 x 16 cm). Electrophoresis was performed under constant current of 2.0 mA per gel for about 18 hrs. The protein was stained with Coomassie Brilliant Blue. The denstmetric tracing of the gels was carried out at 580 mμ.

showed that every strain that underwent mutation at the pap gene by transformation or by NTG treatment revealed that they had lost the character of competence for the transformation.

The cell shapes of the original strain and the transformants with amyR^h were rod, while those of all pap mutants were filamentous. Four to eight cells were linearly linked.

These changes in cell nature brought by the pap gene mutation suggest that the mutation is somehow related to the character of the cell surface.

We prepared membrane fractions from original strain, mutants and transformants with pap by a modified method of Mizushima et al. (7) and analysed their protein components by SDS disc gel electrophoresis (9). Photographs and densitometric tracings of the gels stained by Coomassie Brilliant Blue were illustrated in the Figure. A clear difference was found in the patterns of the gels. A peak (shown by an arrow) contained in the parent (6160-1) was not found either in the mutant (YN-9) nor in the transformant (YY-88). All the other bands in the three preparations were the same. The same difference in the patterns of the membrane fractions were also found between the parent (6160-2) and YN-118 (a pap mutant), YY-110 (a pap transformant).

Although we do not yet know whether the peak is a direct product of the pap^+ gene or not, it is suggested that the changes in productivity of α-amylase and protease, loss of transformability and the changes in cell shape could have some correlation with the loss of this protein in the membrane, because these phenomena were all directed by the single mutation.

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