TRANSFORMATION OF *BACILLUS SUBTILIS* IN POLYGLUTAMATE PRODUCTION BY DEOXY- RIBONUCLEIC ACID FROM *B. NATTO*¹

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Bacillus natto Asahikawa produces a considerable amount of polyglutamate (PGA) and its γ -glutamyltranspeptidase (γ -GTP) activity is 80 times higher than that of the transformable strain *B. subtilis* Marburg which cannot produce PGA. The genetic character of high PGA productivity in *B. natto* Asahikawa was transferred to *B. subtilis* Marburg by the DNA-mediated transformation. Among transformants that acquired high productivity of stringy substance, 4 strains produced a higher amount of PGA. The activities of γ -GTP from the representative 4 transformants were 20 times higher than the activity of *B. subtilis* Marburg. These results suggested the presence of correlation between PGA productivity and γ -GTP activity, and the presence of a specific gene(s) that participated in the production of PGA in *B. natto*.

“Natto,” produced by *Bacillus natto*, is one of the most traditional fermentation foods in Japan and consists of polysaccharide (levan-form fructan) and polyglutamate (PGA). The component of its viscous material is mainly γ -PGA containing D- and L-glutamate in varying proportions. The proportion of D- and L-isomers was found to be dependent on the amount of manganese ion and the kinds of amino acids in the media (1–4). Low concentrations of Mn^{2+} in the growth medium favor the L-isomer while high concentrations result in a polymer containing up to 93% of the D-isomer (4, 5).

D-glutamate is known to be one of the components of the cell membrane and therefore it is very interesting that D- and L-forms of glutamate occur in PGA produced by *B. natto*. A plausible mechanism of the biosynthetic pathway of PGA has been proposed by HOUSEWRIGHT *et al.* (6, 7) for one of the strains

¹ Abbreviations used in this text are as follows: PGA, polyglutamic acid; γ -GTP, γ -glutamyltranspeptidase; *stg*, stringiness.

producing PGA (*B. licheniformis* ATCC 9945A). However, since it is known that various strains differ a great deal in the basic requirements for production of the capsule (1, 8), it is to be assumed that there is more than one biosynthetic pathway.

The productivity of PGA was rather unstable. PGA production was decreased during stock on a nutrient agar slant over long periods. Excretion of glutamate was not only limited to the genus *Bacillus*. Also, PARES *et al.* (9) reported that glutamate was excreted by *Citrobacter intermedium* harboring plasmid DNA.

In this paper, the authors have attempted to clarify the mechanism of "natto" production by using transformation as a genetic technique.

MATERIALS AND METHODS

Microorganisms. Several strains (Asahikawa, E, F and M) of *Bacillus natto* used were isolated from the commercial product, "natto," and the other strain (IFO 3009) was supplied from the Institute for Fermentation, Osaka. *B. subtilis* Marburg 168 (*trp*⁻) was used as the recipient throughout this study.

Media and culture conditions. The compositions of bouillon-yeast extract (BY) medium and of minimal medium used for preparation of competent cells were the same as described by YAMAGUCHI *et al.* (10). The composition of agar plate used for isolation of transformants was sucrose 5%, L-glutamic acid monosodium salt 1.5%, KH₂PO₄ 0.27%, Na₂HPO₄·12H₂O 0.42%, NaCl 0.05%, MgSO₄·7H₂O 0.05% and biotin 0.1 µg/ml, pH 6.4. The medium used for assay of γ-GTP activity consisted of peptone 1.2%, citric acid 0.2%, glycerol 2%, NH₄Cl 0.7%, K₂HPO₄ 0.05%, MgSO₄·7H₂O 0.05%, FeCl₃·6H₂O 0.004% and biotin 0.1 µg/ml, pH 6.8.

Cells were grown in BY-medium with shaking at 30°, 37° or 40° as indicated.

Procedure for transformation. Transforming DNA was prepared from exponentially growing cells by the methods of SAITO and MIURA (11), and filtrated through a Millipore filter (type HA, 0.3 µm) to remove spores. Transformation experiments were performed by the method of YOSHIKAWA (12). As a selective marker, stringiness was taken as the length of strings extricated with a toothpick from the surface of the colonies.

Thin-layer chromatography of the hydrolysis product. Thin-layer chromatography of the hydrolysis products isolated by the method of FUJII (13) was carried out by the ascending technique on cellulose plate at room temperature (Abicell, Funakoshi Chemical Co.) for 2.5 hr. The solvent system used was *n*-butanol-acetic acid-water (60:15:25). After development and drying, the spots were estimated by spraying with methanol containing 0.4% ninhydrin.

Determination of D- and L-glutamate. D-Glutamate was determined by the method of MIYAKE *et al.* (14) with D-amino acid oxidase from hog kidney (Sigma

Chemical Co.). L-Glutamate was determined by the method of UMBREIT and GUNSALUS (15) with L-glutamic decarboxylase from *Escherichia coli* (Sigma Chemical Co.).

Assay of γ -glutamyltranspeptidase activity. γ -Glutamyltranspeptidase (γ -GTP) activity was assayed against γ -glutamyl- α -naphthylamide as a substrate by the method of ORLOWSKI and MEISTER (16). Microorganisms were cultured at 37° for 15 days on a rotatory shaker at 140 rpm. After removing cells by centrifugation, the supernatant solution was used as enzyme solution. A 3-ml volume of 4 mM γ -glutamyl- α -naphthylamide and 50 mM glycylglycine in 48 mM ammonium buffer (pH 8.2) was mixed with 0.05 ml of the enzyme solution. After incubation of the mixture at 37° for 30 min, 1 ml of 1 N HCl containing 3-methyl-2-benzothiazolinohydrazine was added and mixed well. After an appropriate time for incubation at 37°, 0.1 ml of 10 mM ferric chloride was added and the solution was stood at room temperature for 10 min. The absorbance at 550 nm was measured with a spectrophotometer. One unit of γ -GTP activity was defined as the amount of enzyme which liberated 1 μ mol of α -naphthylamine per min at 37° under the above conditions.

RESULTS

Transformation from B. natto to B. subtilis

Transfer of phenotypic tryptophan nonrequirement (*trp*⁺) from *B. natto* Asahikawa to *B. subtilis* 168 by the DNA-mediated transformation was carried out. The cell-DNA mixture was spread on agar plates containing 1.5% L-glutamic acid monosodium salt and 0.1 μ g/ml of biotin. When the DNA concentration was varied, it was observed that transformation frequency rose by increasing the concentration of DNA up to a certain limit, above which no further increase in the frequency was detected. Figure 1 shows the response of the recipient strain *B. subtilis* 168 to DNA preparations from the donor Asahikawa. The frequency of transformants increased linearly with concentration of DNA added, and the saturating concentration of transforming DNA was reached at about 0.1 μ g per ml.

The spontaneous mutation frequency of *trp*⁻ in the recipient strain was less than 10⁻⁸, and the frequency of *trp*⁺ from the DNA-mediated transformation was 7.5 $\times$ 10⁻⁵ (Table 1). When 0.1 μ g per ml of the transforming DNA of strain Asahikawa was employed, it was thought that the double transformation of nutrient requirement and stringiness (*stg*) might be the result of two independent events. The frequency of the double transformation of *trp*⁺ and *stg*⁺ to the transformation of *trp*⁺ was 6 to 7%. Among several strains of *B. natto*, the frequencies of the double transformation of *trp*⁺ and *stg*⁺ to the transformation of *trp*⁺ were 2 to 7%.

TLC of hydrolysis products of the parental strain and the representative transformants

Aliquots of the hydrolysate of the product formed by the parental strain and the representative transformants were analyzed by thin-layer chromatography.

Table 1. Comparison of transformation frequencies among various *Bacillus* strains.

Source of DNA ^a	<i>stg</i> ⁺	<i>trp</i> ⁺	<i>stg</i> ⁺ <i>trp</i> ⁺
Asahikawa	1.1×10^{-1}	7.5×10^{-5}	5.3×10^{-6}
E	1.0×10^{-1}	1.7×10^{-4}	6.3×10^{-6}
F	1.2×10^{-1}	2.7×10^{-4}	5.9×10^{-6}
M	1.0×10^{-1}	8.5×10^{-5}	5.2×10^{-6}
IFO3009	1.1×10^{-1}	1.6×10^{-4}	3.1×10^{-6}
W23	—	3.6×10^{-5}	—

^a DNA concentration used was 0.1 μ g/ml.

These experiments were carried out as described in MATERIALS AND METHODS. After addition of DNA, pancreatic DNase (Boehringer Mannheim GmbH) (10 μ g/ml) was added, followed by incubation for 60 min at 37° and spreading onto agar plates containing L-glutamate and biotin.

Fig. 1. Effect of DNA concentration on transformation frequency.

The recipient strain was grown to competence, as described in MATERIALS AND METHODS. DNA (at the indicated concentrations) isolated from the donor strain was added to these cultures and the mixture was incubated at 37° for 60 min, followed by exposure to deoxyribonuclease (10 μ g/ml) for 60 min at 37°. All samples were plated on minimal media and incubated overnight at 37°, the transformants then being selected. Stringiness was measured after 9 days. Symbols: ○, *stg*⁺; ●, *trp*⁺; ○●, *stg*⁺ *trp*⁺.

Fig. 2. Photo of TLC of hydrolysis products of the parental strain and the representative transformants.

The sample PGA obtained by adding 95% ethanol to the supernatant of culture broth (4:1) was lyophilized. Hydrolysis was performed with 6 N HCl for 1 hr at 100°. Symbols: S, glutamic acid monosodium salt; 1, Asahikawa; 2, transformant 3F1; 3, transformant 8H6; b, before hydrolysis; a, after hydrolysis.

Table 2. Yield of PGA and contents of D- and L-glutamate in PGA from parental strain and its representative transformants.

Strain	PGA (g/l) ^a	D-glu (%)	L-glu (%)
Asahikawa	6.1	85.4	14.6
8H6	0.4	46.7	53.3
3F1	0.6	61.2	38.8

^a PGA as an 80% ethanol-insoluble precipitate was lyophilized after dialysis against water.

The determinations of D- and L-glutamate were performed as described in MATERIALS AND METHODS.

Figure 2 shows a thin-layer chromatogram of products of hydrolysis with 6 N HCl for 1 hr at 100°. As seen from the figure, a highly polymerized compound was produced by transformants as well as by *B. natto* Asahikawa, and glutamate was the sole liberated product of hydrolysis.

Stereoisomers of glutamate in PGA

Capsular PGA from *B. anthracis* contains only D-glutamate, while various strains of *B. subtilis* were found to contain D- and L-glutamate in varying proportions. The contents of D- and L-glutamate in PGA produced by Asahikawa, and representative transformants, 8H6 and 3F1 were determined. As shown in Table 2, glutamate in PGA from the representative transformants is, also, composed of D- and L-glutamate.

*Transfer of high γ -GTP activity from *B. natto* to *B. subtilis**

The authors could observe a high increase of γ -GTP activity in transformants. *B. subtilis* 168 produced about 4 mU of γ -GTP activity per ml in the culture fluid,

Table 3. γ -GTP activity in the culture fluids of the parental strains and the representative transformants.

Strain	γ -GTP activity (mU/ml)
<i>B. natto</i> Asahikawa	476
15A2	16
15A5	15
3F1	34
8H6	39
<i>B. subtilis</i> 168	4

B. natto Asahikawa and transformants were cultured in the medium as described in MATERIALS AND METHODS. After cultivation for 3 days, cells were removed by centrifugation and enzyme activities were then determined.

whereas *B. natto* Asahikawa produced about 470 mU/ml. The difference of the γ -GTP activity between the two strains was correlated with the ability to produce a stringy substance on agar plates containing L-glutamate and biotin. Cells were inoculated on the plate and incubated at 37° for 3 days. *B. natto* Asahikawa, having a stringy substance around its colonies, showed high γ -GTP activity, whereas no stringy substance was detected around colonies of *B. subtilis* 168. Strains of 3F1 and 8H6 among transformants, having a stringy substance around the colonies, showed high γ -GTP activity (Table 3). The amount of PGA produced by strain Asahikawa reached 6.1 g/l. On the other hand, the transformants obtained by the DNA-mediated transformation produced a considerable amount of PGA (0.4–0.6 g/l), in contrast to *B. subtilis* 168 which cannot produce PGA used as the recipient strain (Table 2). This fact suggested the correlation between PGA production and γ -GTP activity.

Time courses of stringiness and γ -GTP activity produced by the parental strains and the representative transformants

The time courses of stringiness and the production of γ -GTP by the parental strains and the transformants were examined. Figure 3 shows time courses of stringiness and γ -GTP activity in *B. natto* Asahikawa, *B. subtilis* 168 and transformants (3F1 and 8H6). In the case of strain Asahikawa used as the donor, stringiness reached the maximum length of about 6 m on agar plate at 37° after 3 days with corresponding maximum γ -GTP activity. Thereafter, stringiness and γ -GTP activity decreased rapidly. In the case of the transformants (3F1 and 8H6), the stringiness increased with the incubation time. The γ -GTP activity of transformants also increased to 124 and 96 mU, respectively. These units showed 21 and 19 fold activities compared to the parental strain, *B. subtilis* 168.

DISCUSSION

PGA was isolated from *B. anthracis* as a capsular substance by IVANOVICS (17, 18), in 1937. PGA was also found to be accumulated in the culture broth of

Fig. 3. Time courses of stringiness and γ -GTP activity of the parental strains and the representative transformants.

Cells were cultured in the medium as described in MATERIALS AND METHODS.

At the times indicated, stringiness (●, ■, ▲, ▼) on agar plates and γ -GTP activity (○, □, △, ▽) in the culture fluids were measured. Symbols: ●○, donor strain *B. natto* Asahikawa; ■□, recipient strain *B. subtilis* 168; ▲△, transformant 3F1; ▼▽, transformant 8H6.

B. subtilis as fermentation product by BOVARNICK (19). Nowadays, the following (1, 20) are recognized as PGA producing strains: *B. subtilis* and its derivatives, *B. licheniformis*, *B. natto*, *B. anthracis*, *B. mesentericus* and *B. megaterium*.

There are many factors connected with the production of PGA by these strains. In particular, culture conditions and the kind of nutrient medium are very important. Depending on these factors, the proportion of D- and L-forms of PGA varied from 40 to 93% (4, 21).

Bacillus natto Asahikawa produced a large amount of extracellular PGA and γ -PGT. *B. subtilis* 168, which produced only a small amount of γ -GTP and cannot produce PGA, was transformed by DNA from *B. natto* Asahikawa. Many transformants having high PGA productivity were isolated. They produced almost 30 to 40% of γ -GTP activity compared with the donor strain. A character of high stringy substance productivity could be transferred from *B. natto* Asahikawa to *B. subtilis* 168 by the DNA-mediated transformation. At high concentrations of transformable DNA of *B. natto* Asahikawa, the proportion of *stg*⁺ to total viable cells was about 30 to 33% and that of *stg*⁺ *trp*⁺ to *trp*⁺ was 27%. This transformation frequency was very high compared with that of nutrient requirement (*trp*⁻ to *trp*⁺) and, in both cases, the inflection points of *stg*⁺, *trp*⁺ and *stg*⁺ *trp*⁺ appeared at 0.1 μ g/ml of DNA concentration after 60 min incubation. Examining transformation frequencies of various *B. natto* strains, no difference between *B. natto* Asahikawa and the other *B. natto* strains was observed. How-

ever, the stringiness was never detected from transformants when *B. subtilis* W23 was used as the donor strains. These results suggested that *B. natto* harbors a different kind of genetic information from *B. subtilis*.

On the TLC plate, a spot of glutamate was detected in PGA produced by transformants (3F1 and 8H6) having stringiness, and an about 20 fold increase of γ -GTP activity in *B. subtilis* 168 used as the recipient strain was obtained. Furthermore, the authors showed the parallelism of γ -GTP activity and stringiness, and concluded that the parental strain and the representative transformants are likely to contain the same gene. Therefore, the increase of γ -GTP activity suggests the presence of a gene(s) which is responsible for the productivity of PGA. The genetic factor(s) of the high PGA production in the DNA donor strain is considered to be transferred to the recipient strain. Since D-glutamate was detected in PGA from transformants, it is suggested that some enzymes such as L-transaminase, racemase or D-transaminase in addition to γ -GTP may be transferred from Asahikawa to its representative transformants by the DNA-mediated transformation. To determine whether the high PGA synthesis is controlled by a single genetic factor or by several, further experiments must be carried out.

On the other hand, it is conceivable that some of the *trp*⁺ mutants would have a pleiotropic effect resulting in altered PGA production. When a high concentration of the DNA from *B. natto* Asahikawa was employed in the transformation experiments, the ratio of the number of *trp*⁺ and *stg*⁺ transformants to *trp*⁺ transformants was 6 to 7% as shown in Fig. 1, whereas the ratio of the number of *stg*⁺ to viable colonies on tryptophan-supplemented SG agar plates was increased to more than 20%. This different ratio might indicate that the *trp*⁺ transformants should be derived from double transformation of independent events.

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