Regulation of Polyglutamate Production in *Bacillus subtilis* (natto): Transformation of High PGA Productivity

Toshio HARA and Seinosuke UEDA

Department of Food Science & Technology, Faculty of Agriculture,
Kyushu University, Hakozaki, Fukuoka 812, Japan

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Bacillus subtilis (natto) produces a considerable amount of polyglutamate (PGA). The genetic character of high PGA productivity of *B. subtilis* (natto) was transferred by DNA-mediated transformation to *B. subtilis* Marburg 168 which cannot produce PGA. The enzyme activity of γ -glutamyltranspeptidase (γ -GTP) of the three transformants, 3F1, F1-9 and M5B4, was 124, 233 and 147 mU/ml, which is about 25, 250 and 100% of that of the donor strains, respectively. However, other enzyme activities such as those of alanine racemase or transaminase among the parental strains and representative transformants were almost the same.

These results suggested that γ -GTP activity might mainly participate in the biosynthesis of PGA in *B. subtilis* (natto).

“Natto,” produced by *Bacillus subtilis* (natto) and steamed soybeans, is one of the traditional fermented foods in Japan and its viscous material consists of polysaccharide (levan-form fructan) and polyglutamate (PGA). The main component of this viscous material is γ -PGA containing D- and L-glutamate in varying proportions.^{1,2)} D-Glutamate is known to be one of the components of the cell wall and therefore it is very interesting that D- and L-forms of glutamate occur in PGA produced by *B. subtilis* (natto). A plausible mechanism for the biosynthetic pathway of PGA has been proposed by Thorne *et al.*^{3,4)} for one of the strains 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,^{1,5)} it is to be assumed that there is more than one biosynthetic pathway.

In our previous papers,^{6,7)} we reported the presence of a specific gene(s) concerned with the production of PGA in *B. subtilis* (natto) and suggested that γ -GTP activity might mainly participate in the biosynthesis of PGA. The present paper describes the mechanism of PGA production in *B. subtilis* (natto) using transformation as a genetic technique.

MATERIALS AND METHODS

Microorganisms. The eight strains of *Bacillus subtilis* (natto) used were isolated from the commercial product, “natto,” and the other ten strains of *B. subtilis* (natto) and *B. subtilis* W23 were supplied by the Institute for Fermentation, Osaka. *B. subtilis* Marburg 168 (*trp*⁻) was used as the recipient strain throughout this study.

Media and culture conditions. The compositions of bouillon-yeast extract (BY) medium and minimal medium used for preparation of competent cells were the same as described by Yamaguchi *et al.*⁸⁾ The composition of agar plate medium used for isolation of transformants was sucrose 5%, L-glutamic acid monosodium salt 1.5%, KH_2PO_4 0.27%, $\text{NaHPO}_4 \cdot 12\text{H}_2\text{O}$ 0.42%, NaCl 0.05%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05% and biotin 0.1 $\mu\text{g}/\text{ml}$, pH 6.4. The medium used for assaying of enzyme activities consisted of peptone 1.2%, citric acid 0.2%, glycerol 2%, NH_4Cl 0.7%, K_2HPO_4 0.05%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05%, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ 0.004% and biotin 0.1 $\mu\text{g}/\text{ml}$, pH 6.8.

Cultivation was carried out on a reciprocal shaker (120 strokes/min, pitch 50 mm) at 30°C or 37°C as indicated.

Procedure for transformation. Transforming DNA was prepared from exponentially growing cells by the method of Saito and Miura,⁹⁾ and filtered through a Millipore filter (type HA, pore size 0.3 μm) to remove spores. Transformation experiments were performed by the method of Yoshikawa.¹⁰⁾ As a selective marker, stringiness (Stg) was taken as the length of string made with a toothpick from the surface of the colonies.

Assay of alanine racemase and transaminase activities. After cultivation overnight in the medium described by Fujii,¹¹⁾ cells were harvested by centrifugation, suspended in 0.9% NaCl and disrupted with a sonic oscillator (Branson Sonifier 185). The sonicated cells were centrifuged and the supernatant solution was used as the crude enzyme solution.

Alanine racemase activity was assayed as the amount of D-alanine converted from L-alanine by the method of Miyake *et al.*¹²⁾ with D-amino acid oxidase from hog kidney (Sigma Chemical Co.) using a Warburg's manometer.

Transaminase activity was determined by the chromatographic assay as follows. The conversion of α -ketoglutaric acid to D-glutamic acid was determined in an incubation mixture which contained the following components in a final volume of 6 ml: M/5 D-alanine 1 ml, M/10 α -ketoglutaric acid 1 ml, 200 μ g/ml pyridoxal-5'-phosphate 1 ml, M/10 phosphate buffer (pH 7.6) and enzyme solution 1 ml. After incubation at 40°C, 20 μ l aliquots from the incubation mixture were applied to Toyo No. 5 filter paper and chromatographed in *n*-butanol-acetic acid-water (4:1:2) for 16 to 18 hr. The chromatograms were dried, and the enzymatically converted product and the converted D-glutamic acid were isolated by cutting out and then determined colorimetrically by the ninhydrin reaction.

Assay of γ -glutamyltranspeptidase activity. Gamma-glutamyltranspeptidase (γ -GTP) activity was assayed against γ -glutamyl- α -naphthylamide (Sigma Chem. Co.) as a substrate by the method of Orlowski and Meister.¹³⁾ Microorganisms were cultured at 37°C for 15 days on a rotatory shaker at 140 rpm. After removing cells by centrifugation, the supernatant solution was used as the 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°C for 30 min, 1 ml of 1 N HCl containing 3-methyl-2-benzothiazolinohydrazone (Sigma Chem. Co.) was added and mixed well. After an appropriate time for incubation at 37°C, 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 is defined as the amount of enzyme which liberates 1 μ mol of α -naphthylamine per min at 37°C under the above conditions.

RESULTS

Biotin requirement and PGA productivity in B. subtilis (natto)

Biotin requirement and PGA productivity in *B. subtilis (natto)* were examined. PGA productivity was represented as stringiness by

measuring the length of string made with a toothpick from the surface of the colonies on agar plates containing L-glutamic acid and biotin. As shown in Table I, biotin was essential for all strains isolated from the commercial product, "natto," for growth. But, most strains of type cultures from the Institute for Fermentation, Osaka, did not indicate biotin requirement for their growth.

Three strains (Asahikawa, F and M) of those listed in Table I, produced greater lengths of string than the others. Table II shows the amount of PGA produced by the above three strains.

TABLE I. BIOTIN REQUIREMENT FOR GROWTH IN *B. subtilis (natto)*

Strain	Biotin requirement
Isolated strain	
Asahikawa	+
D	+
E	+
F	+
G	+
H	+
I	+
M	+
Type culture	
IFO 3009	—
IFO 3013	—
IFO 3022	—
IFO 3023	—
IFO 3024	—
IFO 3025	—
IFO 3037	—
IFO 3108	—
IFO 3335	+
IFO 3339	+

TABLE II. COMPARISON OF PGA PRODUCTIVITY AND STRINGINESS IN *B. subtilis (natto)*

Strain	PGA (g/liter) ^a	Stringiness (m)
Asahikawa	6.1	6
F	3.0	4.5
M	3.1	3

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

TABLE III. COMPARISON OF TRANSFORMATION FREQUENCIES IN VARIOUS BACILLI

These experiments were carried out as described in MATERIALS AND METHODS. After DNA was added, pancreatic DNase (Boehringer Mannheim GmbH) (10 µg/ml) was added and the mixture incubated for 60 min at 37°C and then spread on agar plates containing L-glutamate and biotin.

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}
F	1.2×10^{-1}	2.7×10^{-4}	5.9×10^{-6}
M	1.0×10^{-1}	8.5×10^{-5}	5.9×10^{-6}
IFO 3009	1.1×10^{-1}	1.6×10^{-4}	3.1×10^{-6}
W23	N.D. ^b	3.6×10^{-5}	N.D. ^b

^a DNA concentration was 0.1 µg/ml.

^b Not detected.

Transformation of nutrient requirement from *B. subtilis* (natto) to *B. subtilis*

Transfer of phenotypic tryptophan nonrequirement (*trp*⁺) from *B. subtilis* (natto) to *B. subtilis* Marburg 168 by DNA-mediated transformation was carried out. The cell-DNA mixture was spread on agar plates containing 1.5% L-glutamate and 0.1 µg/ml of biotin.

When 0.1 µg per ml of the transforming DNA of *B. subtilis* (natto) was employed, the frequency of *trp*⁺ in the DNA-mediated transformation was 10^{-5} and that of *stg*⁺ was 10^{-1} (Table III). It was thought that the double transformation of nutrient requirement and stringiness might be the result of two independent events. The frequency of the double transformation of *stg*⁺ *trp*⁺ to the transformation frequency of *trp*⁺ was 7%. Among several strains of *B. subtilis* (natto), the frequencies of the double transformation of *stg*⁺ *trp*⁺ to the transformation of *trp*⁺ were 2 to 7%.

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

B. subtilis Marburg 168 produced about 6 mU of γ-GTP activity per ml of culture fluid, whereas *B. subtilis* (natto) Asahikawa, F and M produced 476, 90 and 157 mU/ml, re-

TABLE IV. γ-GTP ACTIVITY IN THE CULTURE FLUIDS OF THE PARENTAL STRAINS AND REPRESENTATIVE TRANSFORMANTS

Each strain was cultured by the method described in MATERIALS AND METHODS. After cultivation, cells were removed by centrifugation and then enzyme activities were determined.

Strain	γ-GTP activity (mU/ml)
Asahikawa	476
3F1	124
F	90
F1-9	233
M	157
M5B4	142
168	6

spectively (Table IV). The difference of the γ-GTP productivity among these parental strains was correlated with the ability to produce a stringy substance on agar plates containing L-glutamate and biotin. *B. subtilis* (natto), having a stringy substance around its colonies, showed high γ-GTP activity, whereas no stringy substance was detected around colonies of *B. subtilis* Marburg 168. Transformants obtained by the DNA-mediated transformation, having a stringy substance around the colonies, showed high γ-GTP activity.

Other enzyme activities such as those of transaminase or alanine racemase concerned with PGA biosynthesis among the parental strains and the representative transformants were almost the same.

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

The time courses of stringiness and the production of γ-GTP by the parental strains and representative transformants were examined. Figure 1 shows the time courses of stringiness and γ-GTP activity in *B. subtilis* (natto) Asahikawa, *B. subtilis* Marburg 168 and its transformant (3F1). In the case of

FIG. 1. Time Courses of Stringiness and γ -GTP Activity of the Parental Strains and a Representative Transformant.

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 fluid were measured. Symbols: ●○, donor strain *B. subtilis* (natto) Asahikawa; ■□, recipient strain *B. subtilis* 168; ▲△, transformant 3F1.

FIG. 2. Time Courses of Stringiness and γ -GTP Activity of the Parental Strains and a Representative Transformant.

Symbols: ●○, donor strain *B. subtilis* (natto) F; ■□, recipient strain *B. subtilis* 168; ▲△, transformant FI-9. Other details are as in Fig. 1.

Asahikawa used as the donor strain, stringiness reached a maximum length of about 6 meters on an agar plate at 37°C after 3 days and γ -GTP activity also increased linearly during this period. After that, stringiness and γ -GTP activity decreased rapidly. In the case of the transformant (3F1), the stringiness increased with the incubation time. The γ -GTP activity

of the transformant also increased to 124 mU/ml, which showed 21-fold activity compared to the recipient strain, *B. subtilis* Marburg 168. But, this activity became 25% on the basis of that of the donor strain, Asahikawa.

Figure 2 shows the time courses of stringiness and γ -GTP activity in *B. subtilis* (natto) F

FIG. 3. Time Courses of Stringiness and γ -GTP Activity of the Parental Strains and a Representative Transformant.

Symbols: ●○, donor strain *B. subtilis* (natto) M; ■□, recipient strain *B. subtilis* 168; ▲△, transformant M5B4. Other details are as in Fig. 1.

and its transformant, FI-9. Stringiness of the donor strain F reached the maximum length of 4.5 meters but γ -GTP activity was only 90 mU/ml. Transformant FI-9 gave a 1 meter length of string on an agar plate and its γ -GTP activity increased to 233 mU/ml, which corresponded to 2.5-fold compared with that of donor strain F.

On the other hand, the maximum stringiness of donor strain M reached 3 meters on an agar plate at 37°C after 5 days and corresponded to the maximum γ -GTP activity (157 mU/ml). Transformant M5B4 obtained by the DNA-mediated transformation showed high γ -GTP activity but stringiness was very low (Fig. 3).

DISCUSSION

PGA was isolated from *B. anthracis* as a capsular substance by Ivanovics,^{14,15} in 1937. PGA was also found to be accumulated in the culture broth of *B. subtilis* as a fermentation product by Bovarnick.¹⁶ Nowadays, the following^{1,17} are recognized as PGA producing strains: *B. subtilis*, *B. anthracis* and *B. megaterium*. Mutants of *B. subtilis* are sometimes referred to as *B. mesentericus*, *B. subtilis* (natto) and *B. licheniformis*, but the distinctions are not always clear even to microbiologists.¹⁸

According to Bergey's Manual,¹⁹ *B. subtilis* (natto) has been included in *B. subtilis* since the 6th Ed. (1948). In contrast, Amaha *et al.*²⁰ and Kida *et al.*²¹ reported that *B. subtilis* and *B. subtilis* (natto) can be classified whether biotin is essential for growth or not.

As shown in Table I, all isolated strains from the commercial product "natto" required biotin for bacterial growth, but type culture from the Institute for Fermentation, Osaka, did not require biotin for growth with the exception of two strains. This suggested that biotin requirement might be not suitable for distinguishing *B. subtilis* (natto) from *B. subtilis*. In fact, many studies on the genetic as well as biochemical relationship between *B. subtilis* and *B. subtilis* (natto) have revealed that these two bacilli should be considered as one species. Marmur *et al.*^{22,23} reported the success of DNA hybrid formation between the two species and also described the transfer of genetic traits from *B. subtilis* (natto) to *B. subtilis* by DNA. Furthermore, Aoki *et al.*²⁴ reported that transduction as well as transformation from *B. subtilis* to *B. subtilis* (natto) was successful.

As described above, the genetic similarity between *B. subtilis* and *B. subtilis* (natto) was substantiated by the results obtained with the

transformation technique (Table III). However, the transformation frequency of stringiness was very high compared with that of nutrient requirement (*trp*⁻ to *trp*⁺). This might suggest the presence of a specific regulatory gene(s) such as an extrachromosomal element that participated in the production of PGA in *B. subtilis* (*natto*). Pares *et al.*²⁵⁾ reported that glutamate was excreted by *Citrobacter intermedium* harboring plasmid DNA.

The fundamental polymer that is a common component of the cell walls of both Gram-positive and Gram-negative bacteria is called peptidoglycan. The peptide chains in the cell wall of Gram-positive bacteria consist of L-alanine, D-glutamic acid, L-lysine and D-alanine in the primary chain extending from the muramic acid carboxy group. This suggests that amino acid racemase or transaminase exists in the cell membrane of *B. subtilis* (*natto*). Such enzymes have been detected in *B. anthracis*,⁴⁾ *B. subtilis* and *B. licheniformis*.³⁾ Fujii,²⁶⁾ also, detected alanine racemase in *B. subtilis* (*natto*).

Furthermore, Noda *et al.*²⁷⁾ reported that an enzyme (or enzymes) obtained from a cell free extract of *B. subtilis* (*natto*) catalyzed the specific transamidation reaction, for which the dipeptide from D-glutamine was an L-L isomer, while the dipeptide from L-glutamine was an D-D isomer. On the other hand, Troy^{28,29)} showed that a cell envelope particulate fraction of *B. licheniformis* contains a polyglutamyl synthetase which catalyzes the polymerization of L-glutamic acid to form a polymer of γ -D-glutamate. However, Tanaka *et al.*³⁰⁾ reported that D-glutamic acid racemase was not detected in bacilli.

Since it is known that various strains differ a great deal in the basic requirement for PGA production,^{1,5)} it is assumed that there is more than one biosynthetic pathway.^{3,4,28)} To make this point clearer, we investigated enzyme activities concerned with PGA synthesis. Conversion from L-alanine to D-alanine was observed in *B. subtilis* (*natto*) and *B. subtilis*. In addition, the transaminase reaction between D-

alanine and α -ketoglutaric acid to form D-glutamic acid and pyruvic acid was found to occur in both strains. But, we could not detect a significant difference of enzyme activities such as alanine racemase and transaminase between *B. subtilis* (*natto*) which produced a large amount of PGA and *B. subtilis* Marburg 168 which cannot produce PGA (data not shown). However, *B. subtilis* (*natto*) produced a high amount of γ -GTP but *B. subtilis* Marburg 168 produced only a small amount of γ -GTP. Many transformants having high stringiness showed high γ -GTP activities. γ -GTP activity of transformant 3Fl transferred from strain Asahikawa was 124 mU/ml, constituting 25% of the enzyme activity of the donor strain. That of Fl-9 from F was 233 mU/ml corresponding to 2.5-fold that of donor strain F. And, that of M5B4 from strain M was 147 mU/ml, which was equivalent to that of donor strain M. However, the level of γ -GTP activity of transformants was almost equivalent at about 200 mU/ml in spite of a significant difference in γ -GTP activity in each donor strain. This might result from the multiplication of specific regulatory gene(s) concerned with γ -GTP activity in the recipient strain, *B. subtilis* Marburg 168, or the ability of excretion of γ -GTP produced by the specific regulatory gene(s) in the same strain.

In this paper, we showed the parallelism of γ -GTP activity and stringiness, and it is concluded that γ -GTP might participate in the biosynthesis of PGA in *B. subtilis* (*natto*). Otherwise, γ -GTP activity might correspond to that of PGA synthetase reported by Thorne *et al.*^{3,4)} and Troy.²⁸⁾

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