CHARACTERIZATION OF PLASMID DEOXYRIBONUCLEIC ACID IN *BACILLUS NATTO*: EVIDENCE FOR PLASMID-LINKED PGA PRODUCTION¹

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(Received April 6, 1981)

Treatment of *Bacillus natto* (Asahikawa) with acridine orange resulted in the conversion of approximately 64.2% of the population to stringiness negative, which is indicative of the involvement of plasmid deoxyribonucleic acid (DNA). Cleared lysate materials were subjected to agarose gel electrophoresis to determine the plasmid composition of each strain. *B. natto* (Asahikawa) was found to possess a plasmid of approximately 4.2 megadaltons (Mdal). A 4.2 Mdal plasmid has been found to be missing in the stringiness-negative mutants cured by acridine orange. Gamma-GTP activity was also deficient in the *stg*⁻ mutants which were missing the 4.2 Mdal plasmid.

As mentioned in a previous paper (1), a specific regulator gene(s) was reported to be involved in PGA production of *Bacillus natto*. The gene could be transferred from *B. natto* Asahikawa to *B. subtilis* Marburg 168 by the DNA-mediated transformation. Transformants with high productivity of PGA could be obtained at unusually high frequencies.

To investigate the regulatory mechanisms of such exceptional frequencies in *B. natto*, we attempted to analyze genetic elements concerned with the production of PGA. In the present communication, we report evidence that PGA-productivity of *B. natto* may be associated with a specific extrachromosomal element.

MATERIALS AND METHODS

Microorganisms. *B. natto* Asahikawa used in this study was isolated from

¹ Abbreviations used in this text are as follows: AO, acridine orange; γ -GTP, γ -glutamyl-transpeptidase; Mdal, megadalton; PGA, polyglutamate; *stg*, stringiness.

the commercial product "natto." *B. subtilis* IFO 3215 obtained from the Institute for Fermentation, Osaka, was used as reference mobility marker in agarose gel electrophoresis (2).

Media. Sucrose-glutamate (SG) medium for PGA production consisted of: sucrose 50 g, L-glutamic acid monosodium salt 15 g, KH_2PO_4 2.7 g, $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 4.2 g, NaCl 0.5 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g, biotin 100 μg , bacto-agar (Difco) 16 g and deionized water 1 l (pH 6.4). The medium used for assay of γ -GTP activity was composed of peptone 12 g, citric acid 2 g, glycerol 20 g, NH_4Cl 7 g, K_2HPO_4 0.5 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ 0.04 g, biotin 100 μg and deionized water 1 l (pH 6.8).

Acridine orange treatment. Acridine orange (AO, 2:8-bis Dimethylamino-acridine, Eastman Kodak Co.) treatment was carried out by the method of HIROTA (3). A cell density of $10^8/\text{ml}$ at logarithmic phase was inoculated to produce a cell density of about $10^4/\text{ml}$ in nutrient broth containing AO. The culture was incubated overnight at 37° with aeration. Diluted cultures were then plated by spreading on SG agar plates for single colonies which were counted and tested for PGA productivity.

Assay of γ -glutamyltranspeptidase activity. Determination of γ -glutamyltranspeptidase (γ -GTP) activity has been described previously (1). One unit of γ -GTP activity is defined as the amount of enzyme which liberates 1 μmol of α -naphthylamine per min at 37° .

Preparation of cleared lysate. Overnight cultures grown in nutrient broth were inoculated into 200 ml fresh medium and cultured at 30° for 4.5 hr with shaking. Cells were harvested and washed in cold buffer consisting of 0.02 M Tris-HCl (pH 8.0), 0.1 M NaCl and 5 mM EDTA (TES buffer).

Cells were suspended in 3 ml of 25% sucrose in TES buffer. A 0.5-ml portion of EDTA (0.25 M, pH 8.0) and 1.0 ml of lysozyme (5 mg/ml in TES buffer containing 25% sucrose) were added, and the mixture was incubated for 30 min at 37° . A 0.5-ml amount of Pronase E (5 mg/ml in TES buffer predigested for 1.5 hr at 37°) was added, and the incubation was continued for an additional 30 min at 37° . Lysis was brought about at room temperature by the addition of 0.5 ml of 10% sodium dodecyl sulfate in TES buffer. NaCl (0.5 ml, 5 M) was added, and the whole mixture was left overnight at 4° . The mixture was centrifuged for 30 min at $48,000 \times g$ and the supernatant fluid was obtained as cleared lysate.

Agarose gel electrophoresis. Agarose gels were prepared by dissolving 0.7% agarose (Seakem Maline Colloids, Inc.) in TES buffer (pH 7.9) containing 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide in a final concentration. Electrophoresis was carried out at room temperature with TES buffer at 8 V/cm of gel length for 16 hr. All plasmid DNA bands were identified by the fluorescence from integrated ethidium bromide under ultraviolet irradiation (4).

RESULTS

Effect of pH on AO treatment

The elimination of PGA productivity after growth in nutrient broth containing AO (20 $\mu\text{g/ml}$) was examined by taking samples with a toothpick on isolated clones. The results are expressed in terms of elimination rate, *i.e.*, the proportion of clones having become stably stringiness-negative (*stg*⁻) after treatment. The pH influenced the effects of the dye; for example, cell growth was depressed largely in the presence of AO at a high pH but not at pH 7.0, as shown in Fig. 1. After overnight cultivation, the survival rate markedly decreased at high pH ranges

Fig. 1. Effect of pH on the growth of *B. natto* Asahikawa with or without AO.

Overnight culture was inoculated into nutrient broth with or without AO (20 $\mu\text{g/ml}$), and incubated at 37°. Symbols: ○, with AO; ●, without AO.

Table 1. Effect of pH on the survival and elimination rate.

pH	Survival rate (%) ^a	No. of colonies tested	Elimination rate (%) ^b
7.0	47.5	120	6.7
7.2	26.6	120	15.8
7.4	1.5	120	35.8
7.6	0.0006	120	67.5
7.8	0.0003	120	61.2
8.0	0.001	120	55.8

^a Percentage of bacterial population with AO to that without AO after overnight cultivation.

^b Colonies deficient in the property stringiness.

Overnight cultures were inoculated into nutrient broth with AO (20 $\mu\text{g/ml}$) and incubated overnight at 37°. All samples were plated on SG agar plates and colonies were tested for their stringiness with toothpick.

(Table 1). However, the rate of elimination of *stg*⁺ to *stg*⁻ was high at a high pH and the maximum elimination rate was observed at pH 7.6.

Effect of AO concentration on elimination rate

As shown in Fig. 2, the viability of the cells obtained from the treated culture at pH 7.6 decreased remarkably at the concentration of AO 20 $\mu\text{g/ml}$, and at higher concentrations is almost the same. The maximum effective concentration of AO was 20 $\mu\text{g/ml}$ and the elimination rate reached 64.2% (Fig. 3).

All the colonies formed in the untreated control remained *stg*⁺.

Fig. 2.

Fig. 2. Effect of AO concentration on cell growth.

Overnight culture was inoculated into nutrient broth (pH 7.6) containing AO at various concentrations as indicated, and incubated overnight at 37°. Samples were plated on SG agar plates and incubated overnight at 37°. Colonies grown on SG agar plates were then counted.

Fig. 3.

Fig. 3. Effect of AO concentration on elimination rate.

Overnight culture was inoculated into nutrient broth (pH 7.6) containing AO at various concentrations, and incubated overnight at 37°. All samples were plated on SG agar plates and colonies were tested for their stringiness with toothpick after 2 days.

Correlation of biochemical function with plasmid

Plasmids have been known to develop specific functions such as antibiotic resistance, production of bacteriocins and sexual orientation of the host cell, as

well as numerous other functions summarized and reviewed by HELINSKI and CLEWELL (5).

Strain Asahikawa could be eliminated from its PGA productivity by AO

Table 2. Comparison between stringiness and γ -GTP activity of the parental strain and the cured strains.

Strain	Stringiness (cm) ^a	γ -GTP activity (mU/ml) ^b
Asahikawa	600	434
A- 1	0	2.7
A- 2	0	2.8
A- 3	0.5	7.6
A- 4	0	4.6
A- 5	0	4.0
A- 6	0	3.6
A- 7	0	3.8
A- 8	2	28.9
A- 9	0	4.8
A-10	0	3.0
A-11	0	3.6
A-12	0	2.3
A-13	0	3.8
A-14	0	3.9
A-15	0	3.9
A-16	0	4.5

^a Stringiness on SG agar plates was measured by toothpick.

^b Cells were cultured in the medium as described in MATERIALS AND METHODS. After cultivation for 3 days at 37°, cells were removed by centrifugation and enzyme activities in the culture fluids were then determined.

Fig. 4. Agarose gel electrophoresis of cleared lysates of (1) *B. subtilis* IFO 3215 reference mobility plasmid (5.4 Mdal), (2) *B. natto* Asahikawa and (3) *stg*⁻ mutant A-1. Symbols: a, after homogenizing with vortex; b, before homogenizing with vortex.

(20 $\mu\text{g/ml}$, elimination rate was 64.2%). As shown in Table 2, γ -GTP activity of cured strains was decreased. As shown in Fig. 4, strain Asahikawa was found to possess a plasmid of approximately 4.2 megadaltons (Mdal), based on relative mobility of the *B. subtilis* IFO 3215 reference plasmid. However, at least three cured strains derived from the parental strain were missing this plasmid.

These results strongly suggest that this function is determined by the 4.2 Mdal plasmid of strain Asahikawa.

DISCUSSION

Plasmids have been identified in many bacteria (5–7). A large variety of specific biochemical functions such as fertility, resistance to antimicrobial drugs, production of bacteriocins and synthesis of toxins have been attributed to these genetic elements.

Most of the plasmids described in sporeforming bacteria such as *B. pumilus* (8–10), *B. subtilis* (2, 11–13) and *B. megaterium* (10) are cryptic elements lacking genetic markers and are unstable for selection of transformed colonies. However, two cryptic plasmids were found by LOVETT and BRAMUCCI (11). Also, PARES *et al.* (14) reported that glutamate was excreted by *Citrobacter intermedium* harboring plasmid.

The present paper describes the property of plasmid of *B. natto* Asahikawa isolated from commercial product “natto.” PGA production of *B. natto* appears to be governed in part by DNA associated with extrachromosomal elements. A plasmid of approximately 4.2 Mdal has been observed in *B. natto* Asahikawa. Results presented in this communication provide evidence that PGA production may be associated with specific plasmid. The PGA productivity was rather unstable. In strains of *B. natto*, PGA productivity was decreased during stock on nutrient agar slant over long periods and was readily cured when cells were exposed to acridine dye. The agarose gel electrophoretic pattern of plasmid DNA of *B. natto* Asahikawa *stg*[−] strains indicated a positive correlation between the appearance of *stg*[−] phenotype and loss of the 4.2-Mdal plasmid.

Therefore, evidence that PGA-producing ability is plasmid-linked derives from several observations: (i) PGA production is unstable in *B. natto*, (ii) the ability to produce PGA is cured by the use of AO, (iii) a 4.2-Mdal plasmid has been found to be missing in the *stg*[−] mutants and (iv) γ -GTP activity is deficient in the *stg*[−] mutants which are missing the 4.2-Mdal plasmid.

These results strongly suggest that the function of the 4.2 Mdal plasmid of strain Asahikawa is concerned with PGA production and that a specific regulator gene is connected with γ -GTP activity and located on this 4.2-Mdal plasmid.

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