NOTES

Elimination of Plasmid-Linked Polyglutamate Production by Bacillus subtilis (natto) with Acridine Orange

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Treatment of Bacillus subtilis (natto) strains Asahikawa, F, and M with acridine orange resulted in the conversion of approximately 64.2% of the Asahikawa population, 22.4% of the F population, and 9.2% of the M population to polyglutamate-nonproducing colonies. Such curing is suggestive of the involvement of plasmid DNA. Samples of cleared lysates of both parental and their cured strains were subjected to agarose gel electrophoresis to determine the plasmid composition. Parental strains were found to possess a plasmid, but polyglutamate-nonproducing derivatives were missing the plasmid. The plasmid-linked polyglutamate production, which was originally isolated from B. subtilis (natto), could be transformed in B. subtilis.

"Natto," produced by Bacillus subtilis (natto) from soybean, is one of the most traditional fermentation foods in Japan and consists of a polysaccharide (levan-form fructan) and a polyglutamate (PGA). The composition of its viscous material is mainly y-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 ions and the kinds of amino acids in the media (7, 13, 17, 18). Low concentrations of Mn2+ in the growth medium favor the L-isomer, whereas high concentrations result in a polymer containing up to 93% of the D-isomer (7, 20).

In our previous papers (1, 5), a specific regulatory gene(s) was reported to be involved in PGA production by B. subtilis (natto). The gene(s) could be transferred from B. subtilis (natto) Asahikawa to B. subtilis Marburg 168 by DNA-mediated transformation. The frequency of transformation was considerably higher than that observed for a chromosomal marker (trp) used as a control, suggesting a plasmid-related control of PGA synthesis.

To investigate the participation of a plasmid in PGA production, overnight cultures of strains Asahikawa, F, and M, isolated from the commercial product "natto," were treated with acridine orange (AO). Logarithmic phase cultures at a cell density of 10⁶/ml were diluted to produce a cell density of about 10⁴/ml in a nutrient broth at pH 7.6 containing AO at a concentration of 20 μg/ml. The cultures were incubated overnight at 37°C with aeration, during which time the cell density increased to about 10⁷/ml. Diluted cultures were then plated by spreading on SG agar (sucrose 5%, l-glutamic acid monosodium salt 1.5%, KH₂PO₄ 0.27%, Na₂HPO₄ · 12H₂O 0.42%, NaCl 0.05%, MgSO₄ · 7H₂O 0.05%, biotin 0.1 μg/ml-agar [Difco Laboratories] 1.6%, pH 6.4) plates for single colony isolation. After incubation at 37°C for 3 days, colonies were picked up one by one with a toothpick to measure the length of string around colonies, indicating stringiness. After treatment with AO, PGA-nonproducing colonies appeared with frequencies of 64.2% in Asahikawa, 22.4% in F, and 9.2% in M (Table 1). Untreated colonies showed high PGA productivity.

Cleared lysates of B. subtilis (natto) and cured strains were prepared to clarify the presence of plasmid-linked PGA production. Late-logarithmic-phase cells were collected and lysed with lysozyme and sodium dodecyl sulfate, and cleared lysates were obtained according to the method of Guerry et al. (4), with slight modifications. The DNA in the cleared lysates was subjected to electrophoresis on 0.7% agarose gels according to Sharp et al. (14). The sizes of restriction fragments were estimated by using HindIII fragments (21) of lambda phage DNA as standards of molecular weight. The molecular weight of each band was estimated to be 5.7 kilobases. However, no plasmid DNA was de-
TABLE 1. Effect of AO on PGA production

<table>
<thead>
<tr>
<th>Strain</th>
<th>AO concn (µg/ml)</th>
<th>No. of colonies tested</th>
<th>Elimination rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asahikawa</td>
<td>0</td>
<td>120</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>120</td>
<td>64.2</td>
</tr>
<tr>
<td>F</td>
<td>0</td>
<td>120</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>116</td>
<td>22.4</td>
</tr>
<tr>
<td>M</td>
<td>0</td>
<td>120</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>120</td>
<td>9.2</td>
</tr>
</tbody>
</table>

*Colonies deficient in the property of PGA productivity. Overnight cultures were inoculated into nutrient broth (pH 7.6) with or without AO (20 µg/ml) and incubated overnight at 37°C. All samples were plated on SG agar plates, and colonies were tested for their PGA productivity with a toothpick.

Transfer of phenotypic stringiness (stg') from *B. subtilis* (natto) Asahikawa to *B. subtilis* 168 (trp) and Y12S (arg leu) by plasmid-mediated transformation was carried out. Plasmids were prepared by ethanol precipitation of cleared lysate and centrifugation in cesium chloride-ethidium bromide. Transformation was accomplished in both strains, and the levels of stg' were almost similar and surprisingly high (Table 2).

Thus, the following evidence that PGA-producing ability is plasmid linked was accumulated. (i) The ability to produce PGA was cured by AO; (ii) a 5.7-kilobase plasmid was missing in stg' derivatives; and (iii) the plasmid-linked PGA productivity could be transformed in *B. subtilis*.

Most of the plasmids described in spore-forming bacteria such as *B. pumilus* (8–10), *B. subtilis* (2, 3, 6, 9, 15, 16, 19), and *B. megaterium* (10) are cryptic. A few exceptions exist, such as pPL10 and pPL7065, which determine bacteriocin production in *B. pumilus* (11, 12), and pBC7 and pBC16, which determine bacteriocin production and tetracycline resistance, respectively, in *B. cereus* (2).

The results reported here strongly suggest that the function of the 5.7-kilobase plasmid in *B. subtilis* (natto) is concerned with PGA production. This observation suggests that a broader range of functions are associated with plasmid in *Bacillus* strains.

LITERATURE CITED


8. Lovett, P. S. 1973. Plasmid in *Bacillus pumilus* and the...