INACTIVATION OF BLASTICIDIN S
BY BACILLUS CEREUS
II. ISOLATION AND
CHARACTERIZATION OF A
PLASMID, pBSR8, FROM
BACILLUS CEREUS

Sir:
In a recent communication, we described the isolation of Bacillus cereus K55-S1 as a bacterium resistant to blasticidin S (abbreviated to BS), a cytosine-containing antibiotic, and the bacterial conversion of the antibiotic to inactive 4-deamino-4-hydroxy blasticidin S (HO-BS) by the production of BS-deaminase. (Fig. 1)

Enzyme of B. cereus K55-S1, produced in the presence of BS, enabled the cell to grow in a medium containing >500 µg/ml of the antibiotic. This paper concerns the possible involvement of a plasmid in this resistant mechanism.

B. cereus K55-S1 was cultured in peptone water (10 ml, without BS) on a reciprocal shaker at 37°C and was harvested at an optical density of 0.2 (4~5 hours) by centrifugation (8,500 x g, 30 minutes). Cells were washed with saline (0.9 %), suspended in saline solution (0.2 ml), and incubated with 32.5 µg/ml of acriflavine at 37°C for 1 hour (0.1 % of survival rate). After washing twice with saline, the cells were plated on a peptone agar medium and incubated at 37°C for 1 or 2 days. MIC values of 50 isolates against BS were 20 µg/ml for 31 isolates and 50 µg/ml for 15 isolates, and these values were similar to those observed for several B. cereus strains. The remaining 4 isolates showed higher MIC values (>500 µg/ml), which corresponded to those shown by the resistant strains. These results show that resistance of B. cereus K55-S1 was eliminated by the acridine dye treatment, and suggest participation of an extra-chromosomal gene in the production of BS-deaminase.

A recovered sensitive strain, B. cereus K55-S1-A, did not grow in high concentrations of BS nor did it convert the antibiotic.

The above results prompted us to examine if the BS-resistant strain used carried a plasmid. B. cereus K55-S1 strain was cultured overnight at 37°C on a reciprocal shaker in a peptone water medium containing 200 µg/ml of BS, and the cells were harvested by centrifugation (15,000 x g, 30 minutes) and washed twice with TE buffer consisting of 0.01 m Tris-HCl (pH 8.0) and 0.001 m EDTA. Procedures used for the extraction and isolation of the plasmid DNA were essentially the same as those used for Bacillus subtilis plasmids described by Tanaka et al., except for the following modifications; 1) extraction was carried out on a 10~50-ml scale, 2) higher amounts of lysozyme (20 mg/3 ml) and SDS (8 %) were applied.

The prepared DNA showed a faint fluorescent band when agarose gel electrophoresis was performed. The presence of a plasmid was demonstrated by CsCl-EtBr density gradient centrifugation (150,000 x g for 20 hours at 20°C, Beckman VTi 65-2 roter). The plasmid thus obtained (pBSR8) and its Pst I-cleaved product are shown in Fig. 2. From the mobility of the

Fig. 2. Agarose gel electrophoresis of pBSR8.

Lanes: 1; Hind III digested λ DNA, 2; pBSR8, plasmid DNA of Bacillus cereus K55-S1, 3; pBSR8 digested with Pst I.
electrophoresis, the plasmid DNA was estimated to be of 10.5 kb (6.8 Mdalton). The plasmid fraction was separated from others and further purified by a second centrifugation using the same condition. The purified DNA was extracted with phenol-ether solution and was used to the following experiments.

pBSR8 was digested by various restriction endonucleases, and analyses of the fragments indicated the presence of one restriction site for BamHI, BanIII, EcoRV, HindIII, PstI, PvuII and SalI; two sites for BclI and BglII; and no sites for SacI, SmaI or XhoI in the plasmid. Combinations of these enzymes revealed the relative positions of the restriction sites and allowed us to depict the restriction map of pBSR8 as shown in Fig. 3.

In order to prove that this particular plasmid is responsible for the BS-resistance, we introduced pBSR8 into BS-sensitive *B. subtilis* MI112 after ligation with a vector pNC6027 (trimethoprim (TMP)-resistant) at their unique BamHI sites. *B. subtilis* transformants thus obtained were resistant to BS as well as TMP as shown in Fig. 4 and were found to produce BS-deaminase. The plasmid contained in the plasmid DNA was estimated to be of 10.5 kb (6.8 Mdalton). The plasmid fraction was separated from others and further purified by a second centrifugation using the same condition. The purified DNA was extracted with phenol-ether solution and was used to the following experiments.

---

<table>
<thead>
<tr>
<th>Strain</th>
<th>BS sensitivity</th>
<th>Content (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ: <em>B. cereus</em></td>
<td>BS*</td>
<td>200</td>
</tr>
<tr>
<td>K55-S1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>□: <em>B. subtilis</em></td>
<td>BS*</td>
<td>200</td>
</tr>
<tr>
<td>MI112-B79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>■: <em>B. subtilis</em></td>
<td>BS*</td>
<td>0</td>
</tr>
<tr>
<td>MI112</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---: <em>B. cereus</em></td>
<td>BS*</td>
<td>200</td>
</tr>
<tr>
<td>K55-S1-A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---: <em>B. subtilis</em></td>
<td>BS*</td>
<td>200</td>
</tr>
<tr>
<td>MI112</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---
BS- and TMP-resistant transformant had undergone deletion but did carry part of pBSR8. Detailed analyses of BS-resistant gene will be described elsewhere.

Acknowledgment

Authors are grateful to Misses K. Iide, Y. Hijikata and Y. Anzai of this college for their technical assistance.

TOYOSHIE ENDÔ
KAORI KOBAYASHI
Kyoritsu College of Pharmacy,
1-5-30 Shiba-koen, Minato-ku,
Tokyo 105, Japan

NAOHIRO NAKAYAMA
Institute of Applied Microbiology,
The University of Tokyo,
1-1-1 Yayoi, Bunkyo-ku,
Tokyo 113, Japan

TERUO TANAKA
Mitsubishi-Kasei Institute of Life Sciences,
11 Minami-ohya, Machida-shi,
Tokyo 194, Japan

TAKASHI KAMAKURA
ISAMU YAMAGUCHI
Riken, The Institute of Physical and Chemical Research,
2-1 Hirosawa, Wako-shi,
Saitama 351-01, Japan

(Received July 16, 1987)