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

Sir:

In a recent communication[1], we described the isolation of *Bacillus cereus* K55-S1 as a bacterium resistant to blasticidin S (abbreviated to BS), a cytosine-containing antibiotic[2], 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×*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[3] 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[4]. 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×*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.*[5] 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×*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. 1. Deamination of blasticidin S.
Blasticidin S (BS) R=NH_2
4-Deamino-4-hydroxy blasticidin S (HO-BS) R=OH

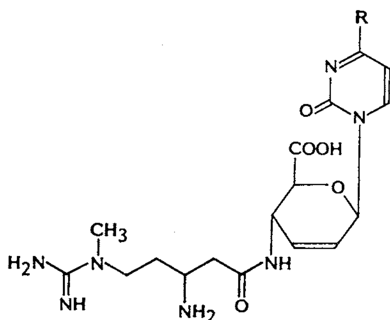


Fig. 2. Agarose gel electrophoresis of pBSR8.
Lanes: 1; *Hind* III digested λ DNA, 2; pBSR8, plasmid DNA of *Bacillus cereus* K55-S1, 3; pBSR8 digested with *Pst* I.

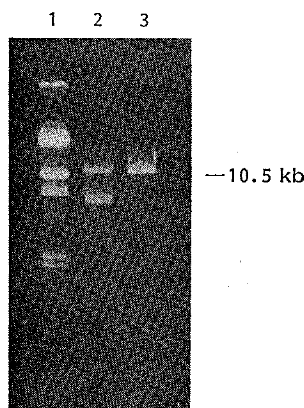


Fig. 3. Restriction endonuclease cleavage map of pBSR8.

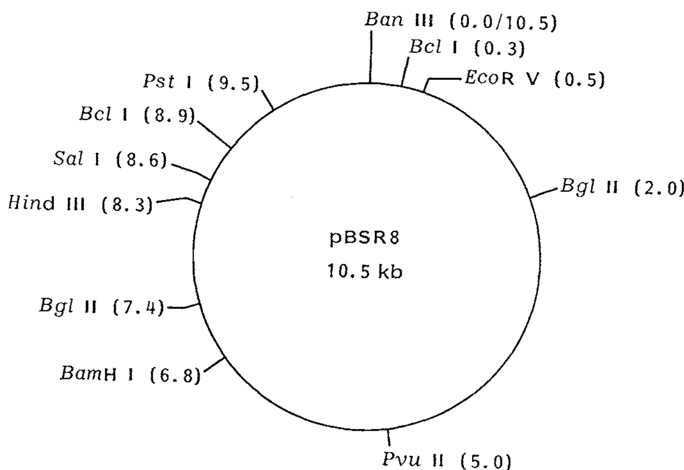
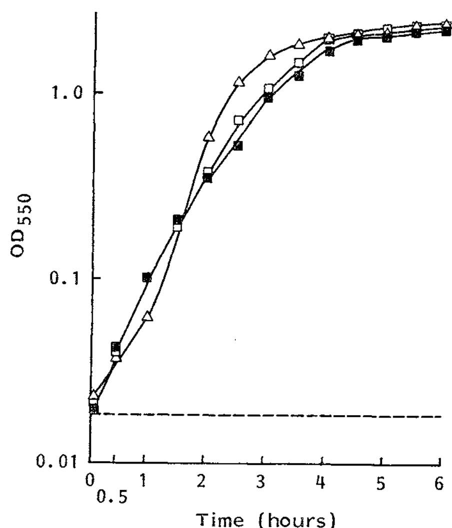


Fig. 4. Growth curves of BS-resistant (BS^r) and BS-sensitive (BS^s) strains. Nutrient broth, 37°C.



	Strain	BS sensitivity	Content (μg/ml)
△:	*Bacillus cereus* K55-S1	BS^r	200
□:	*B. subtilis* MI112-B79	BS^r	200
■:	*B. subtilis* MI112	BS^s	0
---:	*B. cereus* K55-S1-A	BS^s	200
---:	*B. subtilis* MI112	BS^s	200

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 *Bam*H I, *Ban* III, *Eco*R V, *Hind* III, *Pst* I, *Pvu* II and *Sal* I; two sites for *Bcl* I and *Bgl* II; and no sites for *Sac* I, *Sma* I or *Xho* I 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[6] after ligation with a vector pNC602[7] (trimethoprim (TMP)-resistant) at their unique *Bam*H I 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

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

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