INACTIVATION OF BLASTICIDIN S BY *BACILLUS CEREUS* I. INACTIVATION MECHANISM

Sir:

Blasticidin S[1] (abbreviated to BS) is an aminoacyl-nucleoside antibiotic possessing antifungal activity, especially against *Pyricularia oryzae*. After surveying microorganisms degrading the antibiotic in natural circumstances, several authors[2,3] have reported the participation of *Aspergillus* deaminase in microbial inactivation or transformation of this antibiotic and elucidated the detailed kinetics of the enzyme's action[4,5]. This enzyme hydrolyzed the 4-amino group of the cytosine moiety. Based on these results, fungi were believed to be mostly responsible for degradation of the antibiotic in nature.

However, in our attempts to find BS-resistant strains, we isolated two closely related bacteria, designated K55-S1 and K55-S2, which grew on a peptone agar plate containing 2,000 μg/ml of BS. Presence of the bacteria in BS solution caused the loss of UV absorption and of biological activity typically observed when the antibiotic is deaminated.

Isolate K55-S1 is a Gram-positive spore-forming rod, and was considered to be a strain of *Bacillus cereus* from observations of unstained globules in the cells and of an exosporium outside the spores. By comparing the properties summarized in Table 1 with the description of GORDON *et al.*[6] and GIBSON and GORDON[7], the isolate was identified as *Bacillus cereus* Frankland and Frankland, K55-S1.

Isolate K55-S2 shows essentially the same properties as those of K55-S1, except for poor formation of endospores, no growth in a 5%-NaCl medium, and negative peptonization of milk. This isolate was considered to be a natural variant of K55-S1.

B. cereus K55-S1 was cultured at 37°C for 22 hours in a medium composed of peptone 1.0%, NaCl 0.5% and BS 2.0 μg/ml (pH 7.0). BS (200 mg) was added to the culture fluid, washed-cell suspension or crude enzyme extract (100 ml or equivalent) and the mixture was incubated at 37°C until the disappearance of the original spot on a TLC plate and of the 270 nm peak in the UV spectrum.

The reaction mixture was centrifuged (8,500× *g*, 20 minutes) and the supernatant solution was applied to a column of Dowex 50WX8 (1.5× 10 cm). The inactivation products were eluted with 0.5 N NH_4OH and purified by ion-exchange column chromatography using Dowex 50WX2 resin and 1 M pyridine - acetate buffer. In a typical purification, three peaks were detected by ninhydrin reaction and UV absorption. Each fraction, designated as S1-C, -B and -A by elution order, was further purified as the HCl salt using Sephadex G-25. The major product (S1-A, *ca.* 70%) and minor product (S1-C, *ca.* 15%) were identified as 4-deamino-4-hydroxyblasticidin S (HO-BS) and 4-deamino-4-hydroxycytomycin (HO-CM), respectively, by TLC and UV spectral comparison with authentic samples[2]. In its ^1H NMR spectrum (400 MHz spectrometer, DCl as a solvent, Fig. 1), HO-BS gave signals essentially superimposable on those of BS except for two doublets at 7.70 (6H) and 5.96 ppm (5H), which were at 0.2 and 0.4 ppm higher field respectively than those of BS.

The remaining product (S1-B, *ca.* 5%) had the same molecular composition as HO-BS, and its NMR spectrum was identical with those of HO-BS at lower field and of isoblasticidin S (IBS)† at higher field. Consequently, S1-B could be identified as 4-deamino-4-hydroxy-iso-

Table 1. Properties of *Bacillus cereus* Frankland and Frankland, K55-S1.

Rods:	Cylindrical; in chains; 0.9~1.3×1.5~3.0 μm; Gram-positive; unstained globules; long peritrichous flagella; motile.
Spores:	Elliptical; 0.5~0.8×1.2~1.8 μm; central to terminal; exosporium.
Growth temperature:	Maximum 45°C; minimum 10°C; optimum range 20~37°C.
Positive reactions:	Catalase; anaerobic growth; Voges-Proskauer; acid from glucose; growth in 7% NaCl; growth at pH 5.7; peptonization of milk.
Negative reactions:	Acid from arabinose, xylose and mannitol; citrate utilization; nitrate reduction; production of indole; tyrosine decomposition.

† Preparation and characterization of this compound will be published elsewhere.

Fig. 1. ^{1}H NMR spectra of blasticidin S and its analogues (in DCl, 400 MHz, TSP standard).

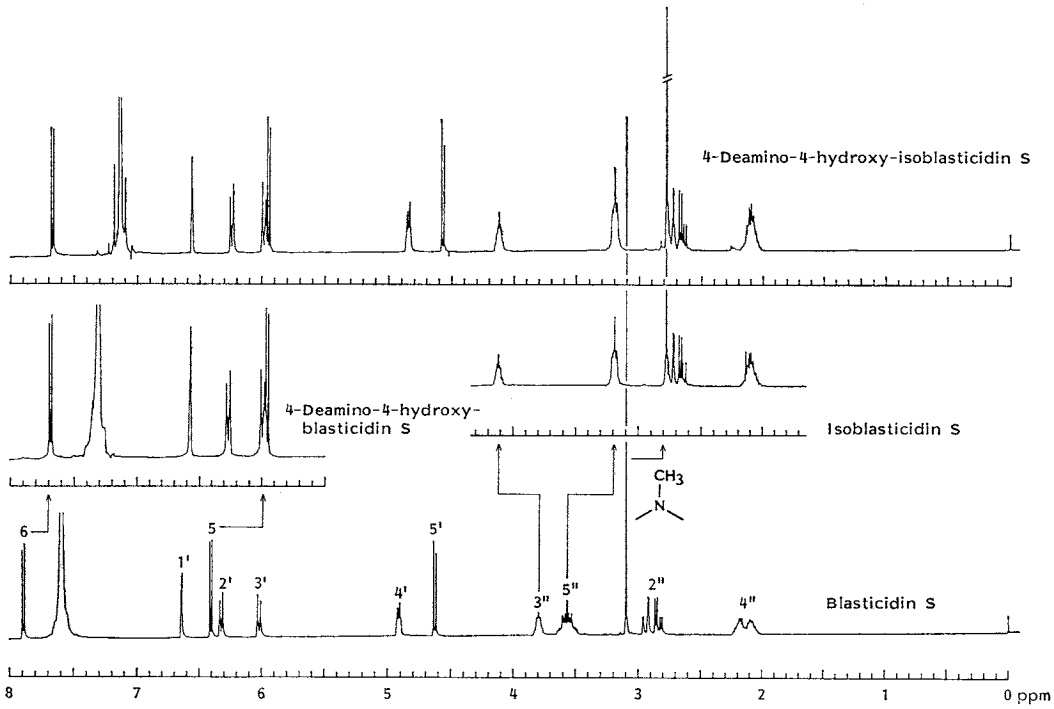
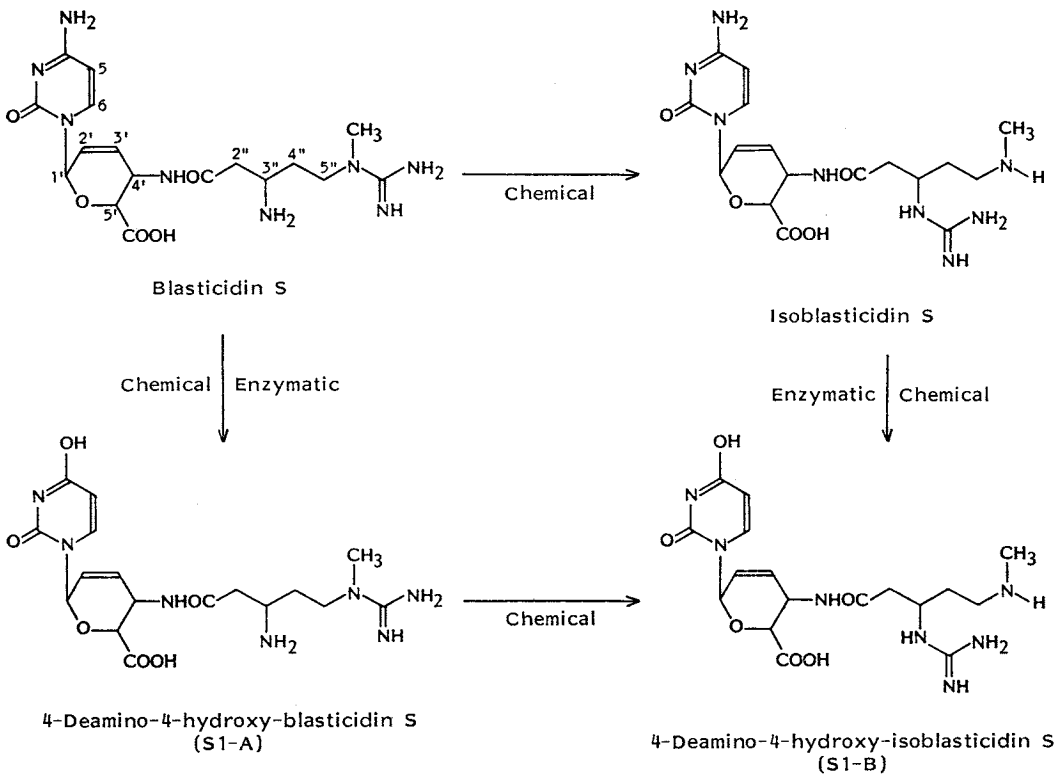


Fig. 2. Scheme for inactivation of blasticidin S.



blasticidin S (HO-IBS).

S1-B (HO-IBS): MP 203~206°C (dec); UV $\lambda_{max}^{H^+}$ nm (ε) 260 (8,500); $\lambda_{max}^{OH^-}$ nm (ε) 261 (8,100).

Anal Calcd for $C_{17}H_{25}O_6N_7 \cdot 2HCl$:
C 41.13, H 5.48, N 14.29.
Found: C 40.73, H 5.49, N 13.93.

Crude extracts of bacterial BS-deaminase liberated from the cells by sonication and centrifugation (15,000×*g*, 30 minutes) was quite labile at 4°C but was stabilized by addition of 10~50% glycerol to the 0.1 M phosphate buffer (pH 8.2). From this extract, an enzyme solution was prepared by ammonium sulfate precipitation (35~65%) and successive chromatography on a DEAE-cellulose column developed with an NaCl gradient (0~0.5 M PBS). In the enzyme solution, BS-deaminase showed optimum activity in the pH range 8~9; its thermal stability followed the tendency, 30>40>50≫60°C (50% glycerol, 10 minutes), and it exhibited deamination activity against BS, IBS and CM, but not against cytidine and arabinosylcytosine. The enzyme solution did not show any isomerization activity (BS to IBS), so the formation of HO-IBS might result from some chemical isomerization process accompanying enzymatic deamination (Fig. 2).

Further enzymatic and genetic studies on this enzyme will be described in the following paper.

Acknowledgments

The authors are grateful to Dr. HARUO SETO of Institute of Applied Microbiology, The University of Tokyo, for his kind supply of authentic deaminated samples and to Dr. KAZUO FURIHATA of the same Institute for the measurement of NMR spectra of inactivation products. We also wish to thank Dr. ISAMU YAMAGUCHI of The Institute of Physical and Chemical Research for his kind advice throughout this work.

TOYOSHIGE ENDŌ
KUMIKO FURUTA
AKIYO KANEKO
TOMO KATSUKI
KAORI KOBAYASHI
AKIKO AZUMA
ATSUKO WATANABE

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

AKIRA SHIMAZU

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

(Received July 3, 1987)

References

1) ŌTAKE, N.; S. TAKEUCHI, T. ENDŌ & H. YONEHARA: Chemical studies on blasticidin S. Part III. The structure of blasticidin S. Agric. Biol. Chem. 30: 132~141, 1966
2) SETO, H.; N. ŌTAKE & H. YONEHARA: Biological transformation of blasticidin S by *Aspergillus fumigatus* sp. Part I. Isolation, characterization and structures of transformed products and their biological properties. Agric. Biol. Chem. 30: 877~886, 1966
3) YAMAGUCHI, I.; H. SHIBATA, H. SETO & T. MISATO: Isolation and purification of blasticidin S deaminase from *Aspergillus terreus*. J. Antibiotics 28: 7~14, 1975
4) YAMAGUCHI, I. & T. MISATO: Active center and mode of reaction of blasticidin S deaminase. Agric. Biol. Chem. 49: 3355~3361, 1985
5) YAMAGUCHI, I.; H. SETO & T. MISATO: Substrate binding by blasticidin S deaminase, an aminohydrolase for novel 4-aminopyridine nucleosidase. Pestic. Biochem. Physiol. 25: 54~62, 1986
6) GORDON, R. E.; W. C. HAYES & C. H. RANG (*Ed.*): The Genus *Bacillus*. (Agricultural Handbook, No. 427). p. 23, pp. 97~99, Agric. Res. Service, U.S. Department of Agriculture, Washington D.C., 1973
7) T. GIBSON & R. E. GORDON: *Genus* I. Bacillus *Cohn* 1872, 174; *nom. gen. cons.* Nomencl. Comm. Intern. Soc. Microbiol. 1937, 28; Opin. A. Jud. Comm. 1955, 39. *In* Bergey's Manual of Determinative Bacteriology. 8th Ed., *Eds.*, R. E. BUCHANAN & N. E. GIBBONS, pp. 529~550, Williams & Wilkins Co., Baltimore, 1974