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Effect of detoxin D on blasticidin S uptake in *Bacillus cereus*[1,2]*

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Summary. The active transport of blasticidin S into the cells of *Bacillus cereus* was significantly inhibited by the addition of detoxin D or poisons of energy metabolism.

Detoxin D is a selective antagonist of blasticidin S, an antibiotic used as a fungicide in the treatment of rice blast disease. The antibiotic activity of blasticidin S is antagonized by detoxin D in *Bacillus cereus*, *Candida albicans*, plants and animals, but not in *Piricularia oryzae* and some other microbes.[3-5] Chemical structures of detoxin D, which contains detoxin D_1 as a main and most active component,[5-7] are entirely distinct from that of blasticidin S[8]. Blasticidin S was reported to inhibit protein synthesis by binding to 50S ribosomal subunits and blocking peptidyltransferase activity in a cell-free system from *E. coli*.[9-11] The inhibition of protein synthesis by blasticidin S in *B. cereus* was reversed by the addition of detoxin D only in intact cells or in protoplasts, but not in cell-free systems, suggesting an effect of detoxin D on blasticidin S transport. The effect of detoxin D on the uptake of ^{14}C-blasticidin S by the cells of *B. cereus* was examined in this paper.

Materials and methods. Detoxin D used in these experiments was a mixture of detoxin D group substances[6,7]; the concentration was kept at 10 μg/ml. ^{14}C-Blasticidin S (labeled by L-methionine-methyl-^{14}C; sp. act. 8.40 mCi/mmole) was supplied by Dr I. Yamaguchi, Institute of Physical and Chemical Research, Wako City 353, Japan.

B. cereus IAM 1729 was cultured in Spizizen's minimal medium[12], supplemented with 1% glucose and 0.2% polypeptone (Daigo) at 37°C. Cells in late-log phase were harvested and washed twice with 33 mM Tris-buffer (pH 7.3), then resuspended in 2% glucose minimal medium without polypeptone at 7 mg dry cells per ml and chilled until use.

The reaction mixture (1 ml) used for the determination of blasticidin S uptake consisted of cell suspension containing various amounts of ^{14}C-blasticidin S and detoxin D or other agents. The cell suspension was preincubated at 37°C for 10 min before the addition of detoxin D and ^{14}C-blasticidin S; if not indicated otherwise detoxin D was added 5 min prior to ^{14}C-blasticidin S. Blasticidin S uptake was followed by removing 0.1 ml aliquots of the reaction mixture and transferring the samples rapidly into 2 ml of ice-cold washing solution, consisting of 150 mM NaCl, 10 mM Tris buffer (pH 7.3) and 0.5 mM $MgCl_2$. The

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cells were collected on a Millipore filter saturated with cold blasticidin S and washed twice with 2 ml of the same solution. Radioactivity on the filter was determined with a liquid scintillation counter (Beckman, Type LS-230) using 5 ml of Bray's solution. The amount of blasticidin S taken up within a given time was determined by radioactivity measurements at the time points shown in figure 1. Initial rate of uptake was measured at 1 min after addition of ^{14}C-blasticidin S. All data are expressed as the mean values of duplicate experiments.

Results and discussion. The time course of blasticidin S uptake by the cells of *B. cereus* is shown in figure 1. When detoxin D was added to the reaction system 5 min prior to the addition of ^{14}C-blasticidin S, the rate of uptake was reduced to 8% of the control value, and consequently low constant cell internal levels were attained. However, when detoxin D was added at the same time as blasticidin S or shortly afterwards, the amount of blasticidin S already taken up by the cells began to decrease and finally the same low basic level was reached. These data suggest that detoxin D caused leakage or efflux of blasticidin S from the cells and that the constant level of blasticidin S within the cells induced by detoxin D corresponded to the equilibrium concentration with the surrounding medium. This assumption is supported by the results shown in figure 2. The level of blasticidin S in the cells was found to be proportional to the concentration of blasticidin S in the medium (figure 2, a). This suggests that blasticidin S is accumulated by the cells

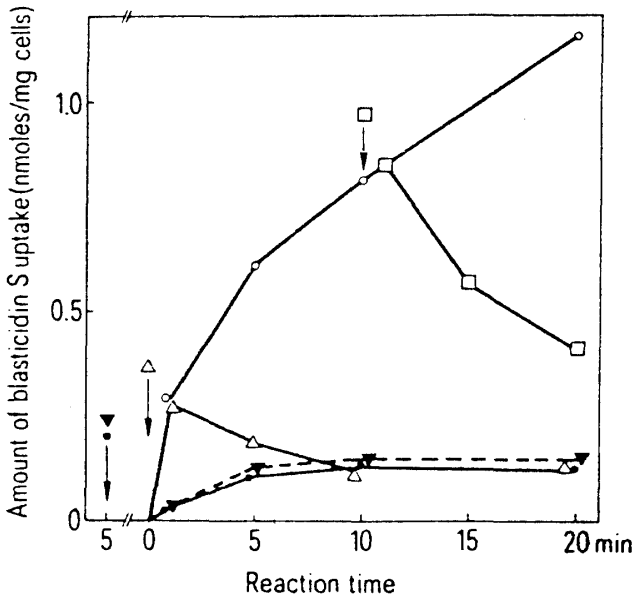

Fig. 1. Effect of deoxin D and sodium azide on blasticidin S uptake. 1.2 mM of ^{14}C-blasticidin S was used for the substrate. Arrows indicate the addition times of detoxin D or sodium azide. Reaction conditions are described in the text. ○ control. ● detoxin D (10 μg/ml). △ detoxin D (10 μg/ml). □ detoxin D (10 μg/ml). ▼ Na N_3 (30 mM).

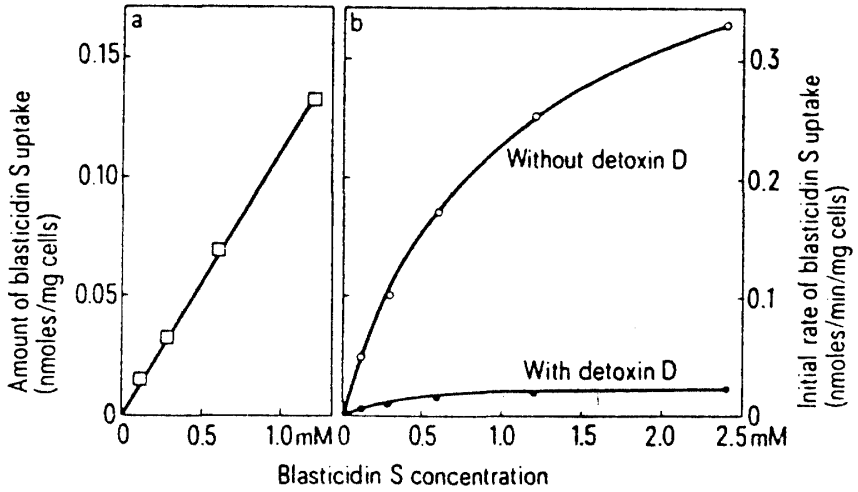

Fig. 2. Blasticidin S uptake at different substrate concentrations *a* amount of uptake at final level induced by deoxin D, *b* initial rate of uptake. ^{14}C-blasticidin S (0.12, 0.3, 0.6, 1.2 and 2.4 mM) was used as substrate. The cells were preincubated with or without detoxin D (10 μg/ml) for 5 min. The amount of uptake was measured in 10 min reaction (a). The initial rate was measured in 1 min reaction (b).

in the absence of detoxin D and that the presence of detoxin D inhibited this transport.

The initial rate of blasticidin S uptake increased with increasing substrate concentration in the presence or absence of detoxin D, approaching a maximum rate of zero order with respect to the concentration of blasticidin S (figure 2, b). In the presence of 10 mM N-ethylmaleimide, a SH-blocking agent of proteins, the amount of blasticidin S taken up within the first 10 min was reduced in the presence and in the absence of detoxin D to 9—9.5% of the control value, which was lower than in the presence of detoxin D only. Preincubation of the cells with detoxin D or poisons of energy metabolism led to a strong reduction of the amount taken up; the reduced uptake was 27.5% with the addition of 10 μg/ml detoxin D, 31.8% with 30 mM sodium azide, 36.3% with 20 mM 2-thenoyltrifluoroacetone and 35.9% with 20 mM 2,4 dinitrophenol (data not shown). The effect of 30 mM sodium azide on the time course of blasticidin S uptake was the same as that obtained with 10 μg/ml detoxin D (figure 1). These results suggest that blasticidin S is taken up by *B. cereus* both by a carrier-mediated passive transport, namely facilitated diffusion, and active transport, and that detoxin D interferes only with the latter.

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2) This is Part VII of "Studies on Detoxin Complex, the Selective Antagonists of Blasticidin S." For Part VI, see the preceding report.

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