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 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 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 ab-

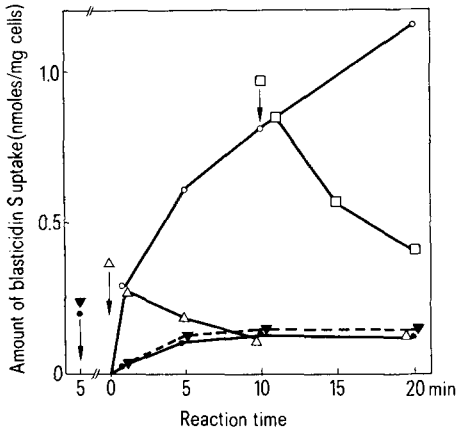

Fig. 1. Effect of detoxin D and sodium azide on blasticidin S uptake. 1.2 mM of ^{14}C-blasticidin S was used for the substrate. Arrows indicate the addition times of detoxin D or sodium azide. Reaction conditions are described in the text. ○ control, ● detoxin D (10 µg/ml), △ detoxin D (10 µg/ml), □ detoxin D (10 µg/ml), ▼ NaN_3 (30 mM).

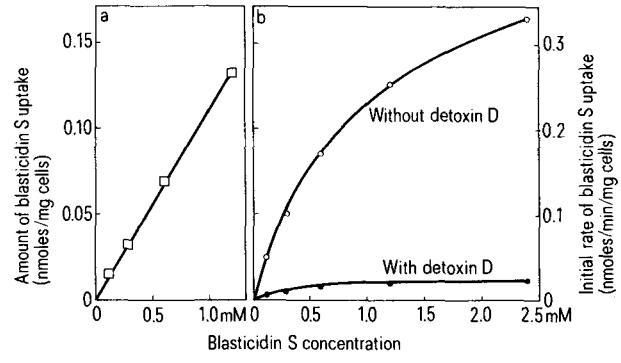

Fig. 2. Blasticidin S uptake at different substrate concentrations *a* amount of uptake at final level induced by detoxin D, *b* initial rate of uptake. ^{14}C-blasticidin S (0.12, 0.3, 0.6, 1.2 and 2.4 mM) was used as substrate. The cells were preincubated with or without detoxin D (10 µg/ml) for 5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

sence 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.

3 H. Yonehara, H. Seto, S. Aizawa, T. Hidaka, A. Shimazu and N. Ōtake, J. Antibiotics *21*, 369 (1969).
4 H. Yonehara, H. Seto, A. Shimazu, T. Hidaka, K. Kakinuma and N. Ōtake, Agr. Biol. Chem. *37*, 2771 (1973).
5 N. Ōtake, H. Seto, K. Kakinuma, A. Shimazu and H. Yonehara, Proc. 1st Intersectional Congr. of IAMS, vol. 5, p. 428. 1975.
6 N. Ōtake, K. Kakinuma and H. Yonehara, Agr. Biol. Chem. *37*, 2777 (1973).
7 K. Kakinuma, N. Ōtake and H. Yonehara, Agr. Biol. Chem. *38*, 2529 (1974).
8 N. Ōtake, S. Takeuchi, T. Endō and H. Yonehara, Agr. Biol. Chem. *30*, 126, 132 (1966).
9 H. Yamaguchi, C. Yamamoto and N. Tanaka, J. Biochem. *57*, 667 (1965).
10 H. Yamaguchi and N. Tanaka, J. Biochem. *60*, 632 (1966).
11 T. Kinoshita, N. Tanaka and H. Umezawa, J. Antibiotics *23*, 288 (1970).
12 J. Spizizen, Proc. natl Acad. Sci. USA *44*, 1072 (1958).

Differential medium for the isolation and identification of *Mycobacterium fortuitum* complex

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Summary. A single medium efficiently selects for and identifies *Mycobacterium fortuitum* complex on the basis of bile tolerance and arylsulfatase activity.

Mycobacterium fortuitum and *M. chelonei* are 2 potentially pathogenic species among the rapidly growing mycobacteria[1]. Because of the similarity of the type of infection caused by them, the 2 species are usually combined under the term *M. fortuitum* complex[2]. Growth on MacConkey agar and arylsulfatase activity are the key tests in identification of *M. fortuitum* complex[3,4].

We have developed a medium which selects for and permits the identification of bile salts tolerant, rapidly growing mycobacteria on the basis of arylsulfatase activity. The medium can be regarded as a modified MacConkey agar in which peptone and bile salts were replaced by tryptose and sodium desoxycholate, respectively. Lactose, neutral red and crystal violet were omitted, and arylsulfatase substrate was incorporated into the medium.

The tryptose-arylsulfate-desoxycholate (TAD) medium was prepared by dissolving 1 g tryptose (Difco Laboratories, Detroit, Mich.), 0.3 g beef extract (Difco), 0.05 g sodium desoxycholate (Difco), 0.5 g NaCl and 1.5 g agar (Difco) in 100 ml distilled water and autoclaving for 15 min at 121 °C. After cooling to 50 °C, 1.5 ml of 0.08 M sterile arylsulfatase substrate solution were added and plates were poured. The complete medium was transparent and colorless. In TAB medium, bile salts No. 3 (Difco) 0.15 g/100 ml medium, were used instead of sodium desoxycholate.

The 0.08 M solution of arylsulfatase substrate was prepared by dissolving 2.6 g phenolphthalein disulfate tripotassium salt (Eastman Kodak, Rochester, NY) in 50 ml distilled water and sterilizing the solution by membrane filtration (Millipore Corp., Bedford, Mass.)[5].

Altogether 45 strains of rapidly growing mycobacteria were used. The various strains were kindly provided by Dr H. Gruft of the New York State Department of Health, Dr I. Weitzman of the New York City Department of Health and Drs McClatchy and Tsang of the National Jewish Hospital, Denver, Colorado. The following type and neo-

The use of tryptose-arylsulfate-desoxycholate agar for the isolation and identification of *M. fortuitum* complex

Species	No. of strains	TAD agar Growth	TAD agar Arylsulfatase	MacConkey agar Growth	MacConkey agar Arylsulfatase*
Mycobacterium fortuitum	18	+	+	+	+
M. chelonei	17	+	+	+	+
M. smegmatis	4	–	–	–	–
M. phlei	3	–	–	–	–
M. vaccae	3	–	–	–	–

* Arylsulfatase test was performed by the CDC method which utilizes liquid substrate medium[5].