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ACCUMULATION OF CELL-BOUND α-AMYLASE IN BACILLUS SUBTILIS CELLS IN THE PRESENCE OF TUNICAMYCIN

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SUMMARY

The effect of tunicamycin on the secretion of extracellular α-amylase by Bacillus subtilis was studied. Tunicamycin at 5μg/ml did not inhibit the synthesis of the enzyme, but in the presence of the drug the cells accumulated to about half of the amount of α-amylase which would have otherwise been secreted into the medium in the absence of the antibiotic. Cell-bound α-amylase accumulated in the presence of tunicamycin reached an amount as much as 10 to 30 times more than that of the control, and was found to accumulate between the cytoplasmic membrane and the cell wall. This effect of accumulation seemed to be specific of tunicamycin.

INTRODUCTION

Tunicamycin (TM), isolated by Takatsuki et al.(1), is a unique antibiotic which contains, as its structural units, uracil, fatty acids, N- acetyl-D-glucosamine and C_{11}-amino-deoxy-dialdose(2). This antibiotic was recently revealed to inhibit the lipid intermediate formation in the process of various complex carbohydrate synthesis in animal cells(3,4), microbial cells(5,6) and plant cells(7). Inhibition by TM of the synthesis of peptidoglycan and teichoic acid of Gram-positive bacteria was reported by us and others(5,6,8,9,10).

We have investigated the synthesis and secretion of extracellular enzymes in Bacillus subtilis cells with the addition

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of this antibiotic(11), and have recently found that a considerable accumulation of cell-bound α-amylase occurred.

Nagata et al.(12) reported the presence of cell-bound α-amylase in this organism and suggested that it might be a precursor to the extracellular enzyme. Gould et al.(13) also reported that there was a small pool of cell-bound α-amylase within the cell wall of Bacillus amyloliquefaciens. The amount of cell-bound α-amylase under normal conditions, however, was very small, and neither conditions that help increase the amount nor mutants that accumulate cell-bound enzyme have been found.

In this report we show that TM specifically caused accumulation of the cell-bound α-amylase in Bacillus subtilis cells, and speculate on this peculiar effect of TM.

MATERIALS AND METHODS

Tunicamycin was dissolved in methanol at a concentration of 1-2mg/ml and stored at -20°C. It was appropriately diluted and added to the medium.

Strains used : B. subtilis NA64(ade⁻, met⁻, amyR2)(14) was used as a TM-sensitive strain. B. subtilis B7(ade⁻, met⁻, amyR2, tmr7) is a TM-resistant and α-amylase hyperproducing mutant derived from B. subtilis NA64. Both phenotypes of B7 resulted from a single mutation(tmr7)(11,15).

Culture conditions : B. subtilis was grown in nutrient broth (Eiken Chem. Ltd., Japan) at 30°C with shaking (120 rev./min.).

Preparation of cell-bound α-amylase : Cell-bound enzyme was prepared after Nagata et al.(12). Cells at given growth stages were harvested and washed three times by centrifugation with 50mM phosphate buffer(pH 6.0) containing 50μg/ml of chloramphenicol. Washed cells were suspended in the same buffer containing lysozyme(200-1000μg/ml) and incubated for 30 to 60 min. at 37°C.

After centrifugation(10,000xg, 5 min.), the supernatant fluid was collected as cell-bound α-amylase preparation.

α-amylase activity was measured by modified Fuwa's method (16) and is indicated in units per the original culture volume.

Fractionation of cells : Cells washed three times as mentioned above were suspended in 50mM phosphate buffer(pH 6.8) containing lysozyme(300μg/ml), 0.5M sucrose and 5mM Mg^{++}, and protoplasts were formed at 30°C. The solution was then centrifuged (15,000xg, 10 min.), and the supernatant fluid was designated as "protoplast wash" fraction.

The protoplasts were suspended and disrupted in 50mM phosphate buffer(pH 6.8) containing 50μg/ml of deoxyribonuclease at 0°C. After removing debris, the supernatant fluid was centrifuged at 100,000xg for 30 min. at 0°C and the precipitates (as

"membrane") were dissolved in 50mM phosphate buffer(pH 6.8). The supernatant fluid was called "cytoplasm".

RESULTS AND DISCUSSION

B. subtilis, a well-known producer of several extracellular enzymes, is very sensitive to TM(1).

When TM was added to the medium at a late logarithmic or early stationary growth phase, B. subtilis NA64 cells accumulated about half the amount of α-amylase which would have otherwise been secreted into the medium in the absence of the drug. In consequence, the amount of extracellular α-amylase was partially repressed.

The concentrations of TM needed for accumulation of cell-bound enzyme were about 1μg/ml for NA64(a TM-sensitive strain) and 50μg/ml for B7(a TM-resistant strain). The B7 strain is a hyperproducer of α-amylase(11) and the amount of the enzyme accumulated in the presence of TM in this strain was also larger than that in NA64.

The increase of cell-bound α-amylase in TM-treated cells began an hour after the addition of TM and reached a plateau 6 or 7 hours later. The amount of cell-bound α-amylase in TM-treated cells became 10 to 30 times larger than that found in control cells which normally had a low and constant value during growth (Fig. 1).

The total activity of α-amylase, that is, extracellular activity plus cell-bound one, in TM-treated cells was almost equal to that in control cells (Fig. 1). This fact shows that TM only interfered with the secretion process without inhibiting the biosynthesis of the enzyme.

The cellular location of accumulated cell-bound α-amylase was studied by fractionating the TM-treated cells. TM was

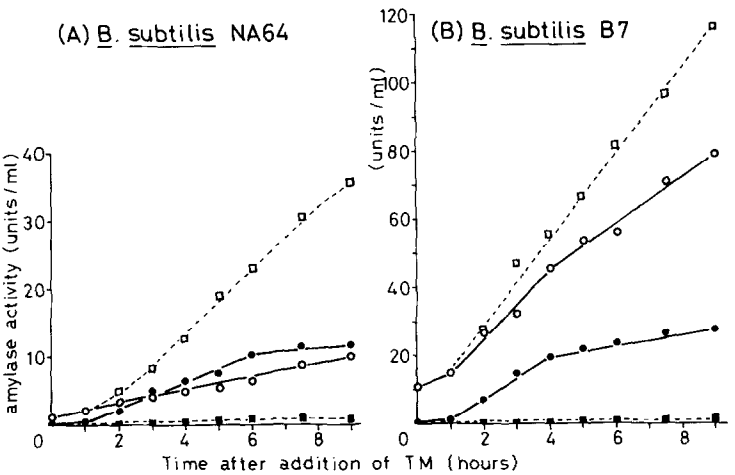

Fig. 1 B. subtilis NA64 and B7 were grown at 30°C. At an early growth phase (OD_{550}=2.0; at time 0), TM was added (5μg/ml for NA64 and 50μg/ml for B7) and at appropriate periods, α-amylase activities of each sample were measured. Control culture did not receive TM.
Growth of TM-treated cells was almost identical to that of the control cells until 8 or 9 hours after addition of TM.
Symbols ; Extracellular α-amylase activity,
o——o TM-treated; □---□ control
Cell-bound α-amylase activity,
●——● TM-treated; ■---■ control

added to the culture at an early stationary growth phase (OD_{550} =2.0) and after 6 hours of incubation, cells were harvested and fractionated as described in "MATERIALS AND METHODS".

As shown in Table 1, more than 90% of the cell-bound α-amylase accumulated with the addition of TM was found in the "protoplast wash" fraction, that is, in the region between the membrane and the cell wall. Moreover, when TM-treated cells were incubated with lysozyme (100 μg/ml) supplemented with 0.6M sucrose, a very rapid release of cell-bound α-amylase into the medium was observed, but such effect did not take place with trypsin or subtilisin (data not shown).

Fig. 2 shows that the effect of TM on accumulation was dependent on the growth phase. B. subtilis NA64 was grown in 500 ml of nutrient broth with shaking and at appropriate intervals, 10 ml of culture broth was taken and divided into two

Table 1. Distribution of α-amylase activity

Fraction	*B. subtilis* NA64 +TM [1]	*B. subtilis* NA64 Control [2]	*B. subtilis* NA64 Increase [1]-[2]	*B. subtilis* B7 +TM [3]	*B. subtilis* B7 Control [4]	*B. subtilis* B7 Increase [3]-[4]
INTRACELLULAR						
"protoplast wash" (A)	13.3	0.38	+12.9 (93.5%)	29.7	0.56	+29.1 (97.3%)
"membrane" (B)	0.03	0.01	+0.02 (0.1%)	0.03	0.02	+0.01 (0.03%)
"cytoplasm" (C)	0.92	0.05	+0.87 (6.3%)	0.80	0.05	+0.75 (2.5%)
(A)+(B)+(C)	14.3	0.44	+13.8 (100%)	30.5	0.63	+29.9 (100%)
EXTRACELLULAR	9.2	25.1	-15.9	60.4	100.0	-39.6

B. subtilis NA64 and B7 were grown to an early stationary growth phase (OD_{550}=2.0) and TM was added (5μg/ml for NA64 and 50μg/ml for B7). After 5 hours' incubation, cells were fractionated as described in "MATERIALS AND METHODS".

Each figure represents the number of units per the original culture volume(ml).

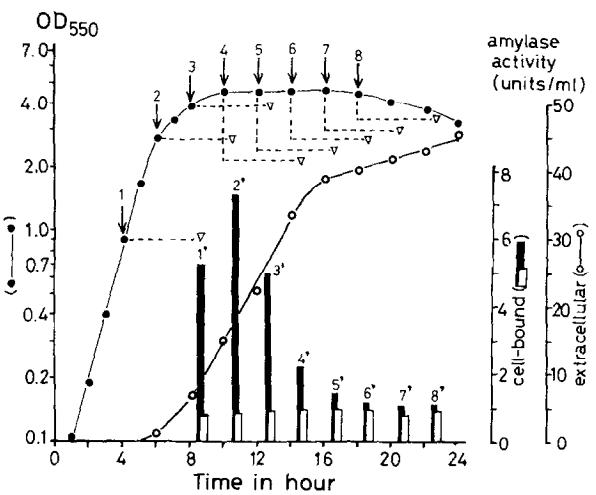

Fig. 2 *B. subtilis* NA64 was grown at 30°C with shaking and the absorbancy (OD_{550}) was measured. At 2-hour intervals, 10 ml culture broth was taken and divided into two portions. One received TM(5μg/ml), and the other did not; Both were further incubated with shaking at 30°C for 4.5 hours and α-amylase activity was measured.

Arrow indicates the time of each addition of TM.

Symbols ; Growth curve(OD_{550}) ●——●

Extracellular α-amylase activity of the original culture o——o

Cell-bound α-amylase activity,

■ TM-treated, □ control

portions. One received TM (5μg/ml), and the other did not; both were further incubated with shaking for 4.5 hours and cell-bound α-amylase activity of each sample was compared. TM exhibited the greatest effect when added to the medium only at a late logarithmic or early stationary phase (e.g., 2 in Fig.2). The antibiotic showed a smaller effect when supplemented after an early stationary phase, although α-amylase production continued actively (e.g., 4 and 5 in Fig. 2).

This suggests that the effect of TM is related to some growth phase-dependent physiological conditions, such as cell wall synthesis or turnover(17).

Nagata et al.(12) reported that cell-bound α-amylase activity could be separated into three components, upon Sephadex G-75 chromatography, referred to as components A, B and C. It was claimed component C was almost similar to the extracellular enzyme trapped in the periplasm of this organism(12). Most of the cell-bound α-amylase accumulated with the addition of TM corresponded to the component C (data not shown), and the accumulated enzyme was not distinguishable from the extracellular one by several criteria including the electrophoretic mobility, as so far studied.

This effect seemed to be specific of TM, because other antibiotics, such as penicillin G, bacitracin, D-cycloserine, chloramphenicol, rifampicin, mitomycin C and polymyxin B showed no similar effect.

From these results, we assume that TM changes the cell wall of B. subtils and accordingly the nascent α-amylase molecules, which penetrated the membrane, may be interfered with when passing across the cell wall and the accumulation of cell-bound enzyme may occur. Or alternatively, the inhibitory effect of

TM on glycosidation of nascent α-amylase molecules may play an important role in this phenomenon.

The reason why other inhibitors of cell wall synthesis did not show a similar effect may be probably explained by the difference in their action mechanisms to that of TM.

The peculiar effect of TM, causing accumulation of cell-bound enzyme to occur, will be useful for studying the biosynthesis and processing of extracellular enzyme, the mechanism of protein passing through the cell wall and also, the role of cell wall in extracellular enzyme secretion.

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