HYPERPRODUCTIVITY OF EXTRACELLULAR α-AMYLASE BY A TUNICAMYCIN RESISTANT MUTANT OF BACILLUS SUBTILIS

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Received February 27,1976

SUMMARY

Several tunicamycin resistant mutants were obtained from Bacillus subtilis NA64. One of them, B7 strain produced a 5-fold larger amount of α-amylase than NA64 did. Only the amount of α-amylase, among excreted proteins, was enhanced. Genetic analyses by transformation suggested that a single mutation in B7 induced both resistance to tunicamycin and hyperproductivity of extracellular α-amylase.

Tunicamycin (TM) is an antiviral antibiotic having a unique mode of action on biosynthesis of virus envelope glycoprotein components(1, 2,3). Recently TM was proved to inhibit the in vitro biosynthesis of polyisoprenol sugar and was suggested to interfere with glycoprotein formation in animal cells(4,5).

TM also shows antimicrobial activity against Gram-positive bacteria, yeasts and fungi(6). In Bacillus subtilis, which is one of the most sensitive bacteria, TM induces spherical or tadpole-shaped cells and thereafter cell lysis(7). TM, moreover, selectively inhibits the incorporation of glucosamine into macromolecules(7). From these observations, TM seems to inhibit the synthesis of cell wall or cell membrane in B. subtilis.

It is anticipated that the cell surface will be altered in TM resistant mutants and productivity of extracellular enzymes may be

changed in some of them. Recently we obtained several TM resistant mutants from B. subtilis NA64. Here we report that B7 strain, one of the resistant mutants, produced a 5-fold larger amount of α-amylase than the parent strain did and that both resistance to TM and hyper-productivity of extracellular α-amylase were caused by a single mutation.

MATERIALS AND METHODS

Crystalline TM (lot T-10) was dissolved in methanol at the concentration of 1 mg/ml and appropriately diluted with methanol or medium.

B. subtilis NA64 (met⁻, ade⁻, amyR2) was used as the parent strain. This strain was derived from B. subtilis 6160 (met⁻, ade⁻, trp⁻, amyR1) by transformation with DNA from B. natto IAM1212 (amyR2). In NA64, the gene amyR1 was replaced by amyR2 and productivity of α-amylase increased 3 to 4 times that of 6160 strain(8).

Mutagenesis was performed after Adelberg's method(9). After treatment of NA64 cells with N-methyl-N'-nitro-N-nitrosoguanidine at 100 μg/ml for 30 min with shaking at 37°C, the cells were collected on a membrane filter, transferred into fresh nutrient broth and grown for 4 hours. 0.1 ml of culture broth was plated onto a nutrient agar plate containing 10 μg/ml of TM and the plates were incubated for 7 days at 37°C. TM resistant mutants were selected from lysis resistant colonies.

α-amylase activity and protease activity were measured by modified Fuwa's method(10) and Hagihara's method(11), respectively.

After incubation for appropriate periods at 30°C, the culture broth was centrifuged (8,000×g, 10 min) and the supernatant fluid was used for enzyme assay and gel electrophoresis. Gel electrophoresis of excreted proteins was performed by the method of Ornstein and Davis (12) and proteins were stained with Coomasie Brilliant Blue.

Extraction of DNA and DNA transformation were performed by Saito and Miura's method(13) and Yoshikawa's method(14), respectively.

RESULTS

We obtained eight TM resistant mutants from B. subtilis NA64. They grew normally in nutrient broth even containing TM up to 10 μg/ml and took the normal rod shape. Productivity of extracellular enzymes was examined. Among the resistant mutants, B7 strain produced a five times larger amount of α-amylase, and another strain (B4) twice larger, but the others were almost at the same level of production as the paren strain. B7 was one of the most resistant mutants which grew almost normally in nutrient broth containing 50 μg/ml of TM (data not shown).

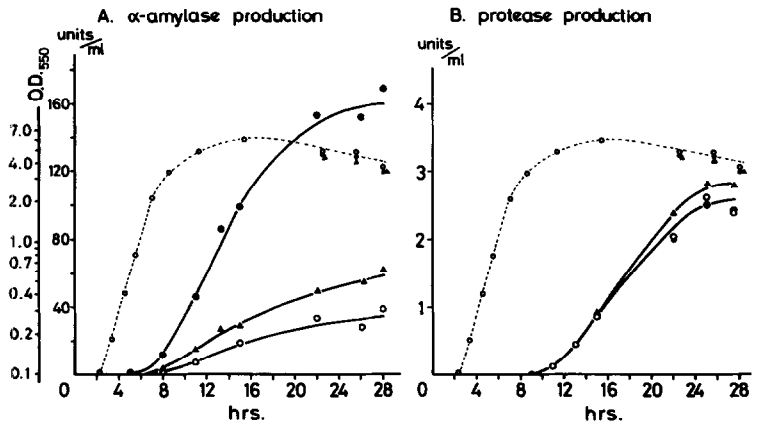

Fig.1. Extracellular enzyme production and growth of TM resistant mutants B7 and B4 in the absence of TM.

Extracellular α-amylase and protease were measured as described in "MATERIALS AND METHODS". Growth was measured by absorbancy at 550 mμ. Symbols: Enzyme activity —o— NA64, —●— B7, —▲— B4
Growth ····o···· NA64, ····●···· B7, ····▲···· B4

As shown in Fig.1A, B7 produced 5-fold α-amylase as compared with the parent strain. In protease production, however, it was just the same as the parent (Fig.1B). B7 as well as B4 produced the same amount of extracellular ribonuclease as NA64 did (data not shown).

It was noted that only the production of extracellular α-amylase was enhanced in B7, as revealed by gel electrophoresis of excreted proteins in culture broth (Fig.2).

α-amylase of B7 migrated at the same position as that of the parent strain as shown in Fig.2 and the optimum pH was about 7 for both enzyme activities. These results suggest that the increased α-amylase activity in B7 culture fluid is attributable to the increased production and/or secretion of α-amylase molecules.

In order to know whether the two phenotypes in B7, i.e., resistance to TM and hyperproductivity of α-amylase, were caused by a single mutation or not, genetical analyses were performed. The parent strain NA64 (amyR2) and B. subtilis 6160 (amyR1) were transformed with DNA

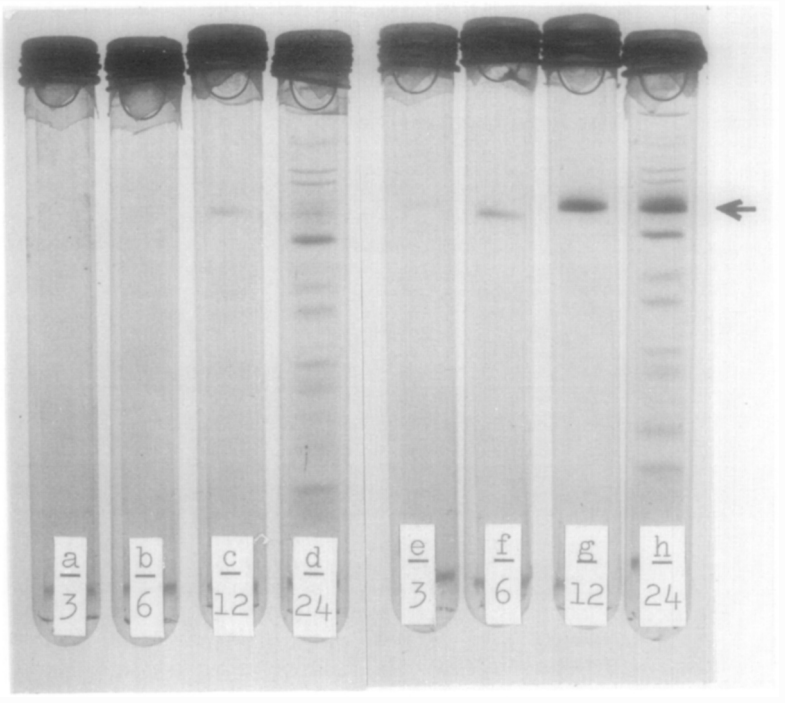

Fig.2. Time course of gel electrophoretic pattern of excreted proteins.

Cells were grown in nutrient broth for 3(a,e), 6(b,f), 12(c,g) and 24(d,h) hours. After centrifugation of the culture broth, 0.1 ml of supernatant fluid was layered on 7.5 % polyacrylamide gel(pH 9.4).
NA64: a,b,c and d B7: e,f,g and h
Arrow indicates the position of extracellular α-amylase protein.
Many protein species that appeared in the culture fluid of 24 hours (d and h) may have been produced by partial cell lysis.

obtained from B7. The recipients NA64 and 6160 normally produced about 35 and 10 units/ml of α-amylase, under the control of amyR2 and amyR1, respectively. TM resistant transformants were selected and productivity of α-amylase was examined.

As shown in Table I, every transformant produced a larger amount of α-amylase than NA64. When B. subtilis 6160 (amyR1) was used as a recipient, it was again observed in every TM resistant transformant that higher productivity of α-amylase than 6160 was transferred.

It was concluded therefore that these two phenotypes in B7 were caused by a single mutation. The gene related to TM resistance was

recipient / units/ml	No. of transformants: NA64 (amyR2)	No. of transformants: 6160 (amyR1)
40 ~ 80	7	20
~120	8	19
~160	7	18
~200	23	8
~240	24	5
~280	14	0
>280	4	0
total	87	70

Table I. α-amylase production of TM resistant transformants.

B. subtilis NA64 and 6160 were transformed by B7 DNA.

TM resistant transformants were selected and grown in nutrient broth supplemented with yeast extract at 30°C for 30 hours in the absence of TM.

α-amylase activity was measured as described in "MATERIALS AND METHODS".

The recipients NA64 and 6160 normally produce about 35 and 10 units/ml of α-amylase, respectively.

The transformants were classified at 40-unit/ml intervals and their number at each class is given.

tentatively named tmr and the mutation in B7 contributing to both TM resistance and hyperproductivity of α-amylase was designated as tmr7.

As a regulator gene for α-amylase production, amyR gene has been confirmed(8). To examine whether tmr7 is a mutation of amyR or not, genetical linkage of tmr7 to aroI116 was studied, because amyR was found to be linked to aroI116(15). The linkage of tmr7 and amyR to aroI116 was compared in terms of transformation of B. subtilis 6160-2 (aroI116$^-$, met$^-$, trp$^-$, amyR1) using DNA from B7 (tmr7, met$^-$, ade$^-$, amyR2).

The co-transfer index of aroI116 and tmr7 was 0.02, while that of aroI116 and amyR was not less than 0.19. From these results, it was concluded that tmr7 was not a mutation of amyR.

DISCUSSION

B7 strain, one of the TM resistant mutants of B. subtilis NA64, produced a 5-fold larger amount of extracellular α-amylase than the

parent strain. The productivity of extracellular protease and ribonuclease by B7 was almost the same as that by the parent strain. Gel electrophoresis of culture fluid showed that only the amount of extracellular α-amylase molecules increased in B7 in comparison with NA64. B7 seems to have acquired peculiar hyperproductivity of α-amylase.

Genetic analyses suggested that these two phenotypes, i.e., resistance to TM and hyperproductivity of extracellular α-amylase, were caused by a single mutation (tmr7). The genetic analyses also revealed that tmr7 was not a mutation of amyR, the regulator gene for α-amylase production.

Recently we found that TM selectively inhibited the formation of lipid intermediate in cell-free peptidoglycan synthesis of Micrococcus lysodeikticus(16). Inhibition of cell wall synthesis in B. subtilis by TM was also reported by Young et al(17).

The hyperproductivity of α-amylase in B7 may be related to the alteration of structure and/or function(s) of cell surface introduced by tmr7.

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