ACETYLATION OF BLASTICIDIN S BY ITS PRODUCING ACTINOMYCETES

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A blasticidin S-producing actinomycetes, *Streptoverticillium* sp. JCM 4673 possesses an enzyme activity which acetylates the drug in the presence of acetyl coenzyme A. The modified drug was biologically inactive when tested against protein synthesis *in vivo* and *in vitro*. Production of the enzyme which acetylates blasticidin S increases with formation of the antibiotic during cell growth.

Organisms which produce antibiotics must be protected from the lethal effect of their own antibiotics.

The resistance mechanisms in streptomycetes which produce antibiotic inhibitors of protein synthesis have been studied with respect to enzymatic inactivation[1~5], resistance of target site[6~8] and membrane permeability[4,9].

Certain microorganisms producing aminoglycoside antibiotics such as streptomycin and neomycin, do not possess drug-resistant ribosomes; rather, antibiotic-modifying enzymes play the main role of self-protection[3~5,10~12]. Other aminoglycoside antibiotic-producers which do not possess such enzymes, have ribosomes resistant to the toxic products[13,14].

A kanamycin-producing strain, *Streptomyces kanamyceticus* expresses a kanamycin-acetylating enzyme[15]. HOTTA *et al.*[12], reported that the enzyme played a major role in the resistance mechanism. However, it was recently demonstrated by others that self-resistance in this strain is attributable to ribosomal resistance acquired with formation of kanamycin[16]. Thus, various mechanisms of protection can be found in microorganisms which produce the same group of antibiotics. Such observation prompted us to examine self-protection mechanisms in microorganisms which produce other types of antibiotics.

Puromycin, one of the nucleoside antibiotics, inhibits protein synthesis by substituting for aminoacyl t-RNA and serving as an acceptor for the nascent peptide chain of ribosome-bound peptidyl t-RNA[17]. We and PÉREZ-GONZÁLEZ *et al.*, reported recently that a puromycin-producing organism, *Streptomyces alboniger*, possessed an enzyme which acetylated the 2″-amino group of *O*-methyltyrosine moiety of a puromycin molecule in the presence of acetyl coenzyme A[18,19] and puromycin 2″-*N*-acetyltransferase was purified[20]. We demonstrated that by this means, the producing organism could overcome the toxic effects of the drug[20].

Blasticidin S is another nucleoside antibiotic; like puromycin, it inhibits peptide-chain elongation in bacterial and mammalian cells[21,22].

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In the present study, we show that a strain of blasticidin S-producing actinomycete possesses an activity that inactivates the drug in the presence of acetyl coenzyme A. The substrate specificity of the enzyme and the time course of the production of blasticidin S and blasticidin S-acetylating enzyme were also investigated.

Materials and Methods

Bacterial Strains, Media and Growth Conditions

Streptoverticillium sp. JCM 4673 (=*Streptomyces morookaensis* KCC S-0673) was used as blasticidin S-producing microorganism. This strain is also known to produce 8-azaguanine. *Bacillus cereus* IFO 3001 was used to assay blasticidin S activity by an agar diffusion method; this bacillus was tolerant up to 100 μg/ml of 8-azaguanine. *Streptomyces griseus* KSN[3~5] and *Streptomyces flavotricini* subsp. *pseudochromogenes* V-13-1[23] were used as strains sensitive to blasticidin S.

Stock cultures of these actinomycetes were maintained on medium containing, per liter, carbon source 10 g, Polypepton 2 g, yeast extract 1 g, meat extract 1 g and agar 20 g, pH 7.0. In the case of the JCM 4673 and KSN strains, fructose was used as carbon source, and glucose was used for growth of the V-13-1 strain. Liquid medium, designated GMP medium, contained per liter, glucose 10 g, NaCl 5 g, Polypepton 4 g, yeast extract 2 g, meat extract 2 g and $MgSO_4 \cdot 7H_2O$ 0.25 g, pH 7.0. Bacterial strains were grown in GMP medium at 28°C to a given time in a jar fermentor or a shaking flask.

Blasticidin S Tolerance of the Producing-organism *In Vivo*

One loopful of spore mass was inoculated into 10 ml GMP medium and grown at 28°C for 24 hours. A sample (0.1 ml) of this culture broth was transferred to 10 ml of the same medium containing a given concentration of blasticidin S hydrochloride. Growth was measured after incubation at 28°C for 24 hours.

Preparation of Ribosomes and S-150 Fraction

This was performed according to the methods of SUGIYAMA *et al.*[5], using mycelia grown in GMP medium at 28°C to the late exponential phase of growth.

Cell-free Protein Synthesis

Polyuridylate-directed polyphenylalanine synthesis *in vitro* was carried out according to SUGIYAMA *et al.*[5].

Assay of Blasticidin S-inactivating Activity

The blasticidin S-inactivating enzyme (blasticidin S acetyltransferase) was measured in a reaction mixture (200 μl) consisting of 80 mM Tris-HCl (pH 7.65), 10 mM magnesium acetate, 6 mM 2-mercaptoethanol, 2 mM acetyl coenzyme A, 100 μg blasticidin S hydrochloride and S-150 fraction from *Streptoverticillium* sp. JCM 4673. The mixture was incubated at 28°C for 1 hour, then heated at 70°C for 10 minutes, followed by centrifugation at 4,700$\times g$ for 10 minutes. A 100-μl portion of the supernatant was placed in a well in an agar plate overlayered with spores of *B. cereus* IFO 3001. Blasticidin S-inactivating activity was measured by the residual antibacterial activity of the drug after acetylation. One unit of enzyme is that activity which acetylates 1 μmol of blasticidin S in 1 hour.

Results and Discussion

Initial experiments (data not shown) indicated that *Streptoverticillium* sp. JCM 4673 was tolerant to blasticidin S hydrochloride to over 100 μg/ml, when cultivated in GMP medium. In contrast, *S. griseus* KSN and *S. flavotricini* subsp. *pseudochromogenes* V-13-1 were susceptible to 40 μg/ml of the drug under identical growth conditions. This suggested that strain JCM 4673 possessed resistant mechanism(s) to its own antibiotic product.

In the present study, the ribosomes and S-150 fraction from strain JCM 4673 were prepared by

Fig. 1. Effect of blasticidin S on polyphenylalanine synthesis by an *in vitro* system consisting of the ribosomes of *Streptoverticillium* sp. JCM 4673 and the S-150 fraction of *S. griseus* KSN.

The mixture contained ribosomes (60 μg) and the S-150 fraction (240 μg as protein) and polyphenylalanine synthesis was carried out at 28°C for 20 minutes in the presence or absence of blasticidin S hydrochloride.

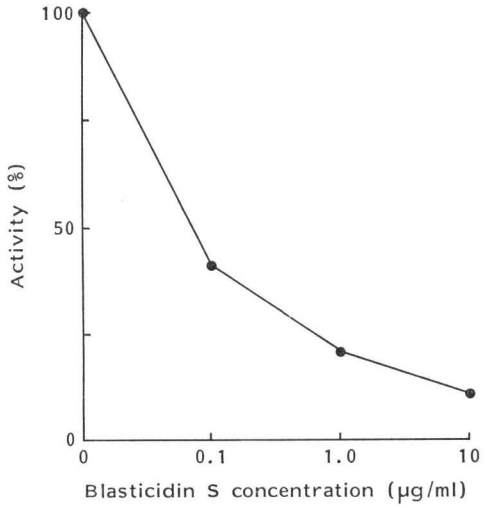


Fig. 2. Inactivation of blasticidin S by the S-150 fraction from *Streptoverticillium* sp. JCM 4673.

Reaction mixtures (200 μl) contained 80 mM Tris-HCl (pH 7.65), 10 mM magnesium acetate, 6 mM 2-mercaptoethanol and 100 μg blasticidin S hydrochloride together with one of the following (1~6).

1: Buffer I (10 mM Tris-HCl (pH 7.65), 10 mM magnesium acetate, 6 mM 2-mercaptoethanol and 30 mM NH_4Cl), 2: S-150 fraction (480 μg as protein) from JCM 4673 strain, 3: S-150 fraction and acetyl coenzyme A (2 mM), 4: S-150 fraction and ATP-Na_2 (2 mM), 5: buffer I and acetyl coenzyme A, 6: buffer I and ATP-Na_2.

The mixture was incubated at 28°C for 1 hour and then at 70°C for 10 minutes. A 100-μl portion of supernatant obtained by a low speed centrifugation was subjected to the antibiotic assay using *Bacillus cereus* IFO 3001 as a test organism.

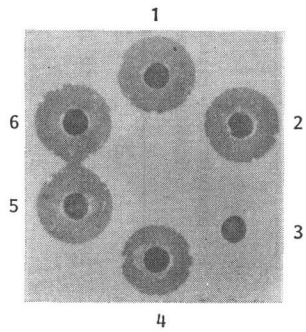


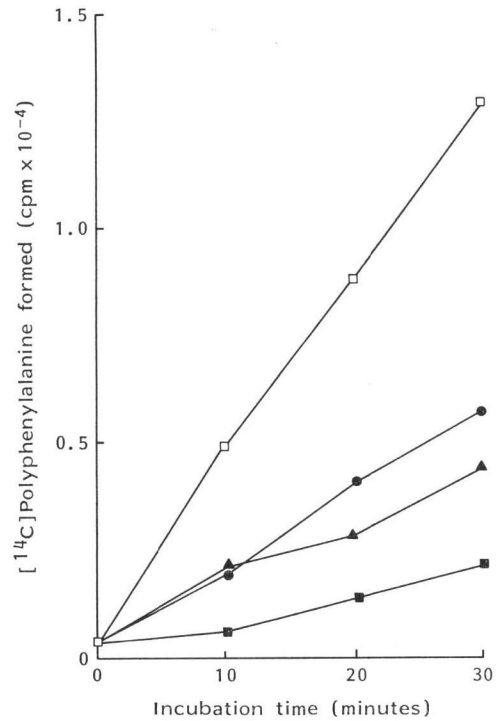
Fig. 3. Protection of polyphenylalanine synthesis against blasticidin S inhibition.

Four μl of a 500 μg/ml blasticidin S hydrochloride solution was incubated for 1 hour at 28°C with the S-150 fraction (51 μl, 480 μg as protein) from *Streptoverticillium* sp. JCM 4673 strain, in the presence or absence of 20 μl of 20 mM acetyl coenzyme A (20 mM).

The reaction mixture (75 μl) was heated at 70°C for 10 minutes and centrifuged to remove precipitate.

A 37.5 μl portion of the resulting supernatant was added to the polyphenylalanine-synthesizing system containing ribosomes from JCM 4673 strain and the S-150 fraction from *S. griseus* KSN.

Additions are as follows: □; buffer I (37.5 μl), ●; S-150 fraction from JCM 4673 strain incubated in the absence of blasticidin S hydrochloride and of acetyl coenzyme A, ▲; S-150 fraction incubated with blasticidin S and acetyl coenzyme A (2 mM), ■; S-150 fraction incubated with blasticidin S.



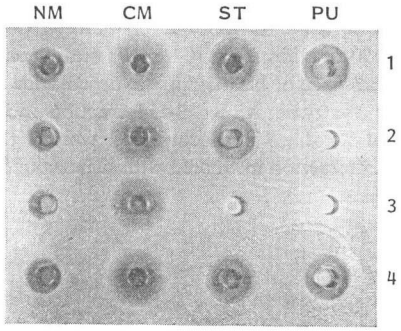
using protease inhibitors to avoid possible degradation of ribosomes during handling[5]. Nevertheless the polyuridylate-directed polyphenylalanine-synthesizing activity was low. We therefore reconstituted a heterogeneous polyphenylalanine-synthesizing system consisting of the ribosomes

Fig. 4. Substrate specificity of blasticidin S acetyltransferase activity.

Reaction mixtures (200 μl) contained 80 mM Tris-HCl (pH 7.65), 10 mM magnesium acetate, 6 mM 2-mercaptoethanol and each antibiotic substrate together with one of the following (1~4).

1: Buffer I, 2: S-150 fraction from JCM 4673, 3: S-150 fraction and acetyl coenzyme A (2 mM), 4: buffer I and acetyl coenzyme A. Antibiotic assay was carried out as described in the legend to Fig. 1.

Substrate antibiotics added to the reaction mixture were 100 μg neomycin sulfate (NM), 60 μg chloramphenicol (CM), 200 μg streptothricin sulfate (ST) or 200 μg puromycin dihydrochloride (PU).



from strain JCM 4673 and the S-150 fraction from *S. griseus* KSN. As a result, an efficient polyphenylalanine-synthesizing activity was observed (data not shown), indicating that the ribosomes of strain JCM 4673 were active, and suggesting that the S-150 fraction from the strain may contain some inhibitory factor. This heterogeneous system was used to investigate the effect of blasticidin S on polyphenylalanine synthesis. As shown in Fig. 1, polyphenylalanine synthesis was inhibited by 0.1 μg/ml blasticidin S, indicating that the ribosomes of the blasticidin S producer were highly susceptible to inhibition by its antibiotic product. This suggests that the strain must have blasticidin S-resistant mechanism(s) other than ribosomal resistance.

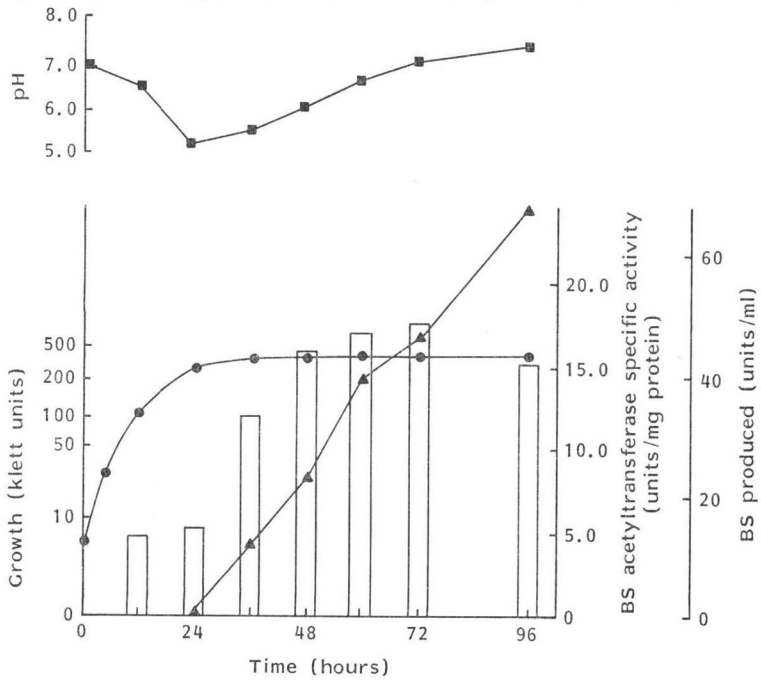
We then examined the presence of drug-inactivating activity in cell extracts of the blasticidin S producer. The activity of blasticidin S disappeared after incubation with acetyl coenzyme

Fig. 5. Time course of blasticidin S acetyltransferase production by *Streptoverticillium* sp. JCM 4673.

The JCM 4673 strain was grown in GMP medium. The specific activity of the enzyme was expressed as unit (see method) per mg protein.

●; Growth, ■; pH, ▲; amount of blasticidin S (BS) produced in the broth, vertical bars; specific activity of BS acetyltransferase.

One mg of blasticidin S hydrochloride used in this study contains 880 units as acid-free antibiotic.



A and the S-150 fraction from strain JCM 4673 (Fig. 2). Substitution of ATP for acetyl coenzyme A was ineffective. The inhibitory effect of blasticidin S on the heterogeneous polyphenylalanine-synthesizing system (see above) decreased in the presence of acetyl coenzyme A (Fig. 3), showing that the blasticidin S-acetylating activity could protect the protein synthesizing machinery in strain JCM 4673.

It is known that several antibiotics are inactivated by acetylation[15,18,24~26]. We examined whether the S-150 fraction from strain JCM 4673 could acetylate other antibiotics. Fig. 4 shows that the fraction inactivates strongly streptothricin and puromycin, in the presence of acetyl coenzyme A. However, it should be pointed out that puromycin was completely inactivated by incubation with the S-150 fraction in the absence of added acetyl coenzyme A. Thus, the S-150 fraction appears to have a puromycin-inactivating activity that does not involve acetylation. Weak inactivation of neomycin by the S-150 fraction was observed in the absence of acetyl coenzyme A.

It is known that the production of a streptomycin-inactivating enzyme, streptomycin 6-phosphotransferase, increases with the formation of streptomycin[27] during growth and it was of interest to investigate whether the syntheses of blasticidin S-acetylating enzyme and blasticidin S were linked. Fig. 5 shows the time course of growth and production of blasticidin S and blasticidin S acetyltransferase activity. The production of the enzyme increased with formation of the antibiotic, suggesting that the enzyme was induced by blasticidin S accumulated in the broth. However, we can not exclude other possibilities, such as an auto-inducer.

There remains the question of whether acetylation is involved in the biosynthesis of blasticidin S in the producing organism. Determination of the site of acetylation, studies on the physico-chemical properties of the enzyme, and of the puromycin-inactivating factor are in progress.

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References

1) BENVENISTE, R. & J. DAVIES: Aminoglycoside antibiotic-inactivating enzymes in actinomycetes similar to those present in clinical isolates of antibiotic-resistant bacteria. Proc. Natl. Acad. Sci. U.S.A. 70: 2276~2280, 1973
2) SKINNER, R. H. & E. CUNDLIFFE: Resistance to the antibiotics viomycin and capreomycin in the *Streptomyces* species which produce them. J. Gen. Microbiol. 120: 95~104, 1980
3) SUGIYAMA, M.; H. MOCHIZUKI, O. NIMI & R. NOMI: Roles of streptomycin 6-kinase and ribosomal affinity to streptomycin in self-protection of streptomycin producer. Biotechnol. Lett. 3: 357~362, 1981
4) SUGIYAMA, M.; H. MOCHIZUKI, O. NIMI & R. NOMI: Mechanism of protection of protein synthesis against streptomycin inhibition in a producing strain. J. Antibiotics 34: 1183~1188, 1981
5) SUGIYAMA, M.; M. SAKAMOTO, H. MOCHIZUKI, O. NIMI & R. NOMI: Purification and characterization of streptomycin 6-kinase, an enzyme implicated in self-protection of a streptomycin-producing micro-organism. J. Gen. Microbiol. 129: 1683~1687, 1983
6) CUNDLIFFE, E.: Mechanism of resistance to thiostrepton in the producing-organism *Streptomyces azureus*. Nature 272: 792~795, 1978
7) CUNDLIFFE, E. & J. THOMPSON: The mode of action of nosiheptide (multhiomycin) and the mechanism of resistance in the producing organism. J. Gen. Microbiol. 126: 185~192, 1981
8) THOMPSON, J.; E. CUNDLIFFE & M. J. R. STARK: The mode of action of berninamycin and the mechanism of resistance in the producing organism, *Streptomyces bernensis*. J. Gen. Microbiol. 128: 875~884, 1982
9) MALIK, V. S. & L. C. VINING: Chloramphenicol resistance in a chloramphenicol-producing *Streptomyces*.

Can. J. Microbiol. 18: 583~590, 1972

10) SUGIYAMA, M.; H. KOBAYASHI, O. NIMI & R. NOMI: Susceptibility of protein synthesis to streptomycin in streptomycin-producing *Streptomyces griseus*. FEBS Lett. 110: 250~252, 1980
11) SUGIYAMA, M.; O. NIMI & R. NOMI: Susceptibility of protein synthesis to neomycin in neomycin-producing *Streptomyces fradiae*. J. Gen. Microbiol. 121: 477~478, 1980
12) HOTTA, K.; H. YAMAMOTO, Y. OKAMI & H. UMEZAWA: Resistance mechanisms of kanamycin-, neomycin- and streptomycin-producing streptomycetes to aminoglycoside antibiotics. J. Antibiotics 34: 1175~1182, 1981
13) YAMAMOTO, H.; K. HOTTA, Y. OKAMI & H. UMEZAWA: Self-resistance of a *Streptomyces* which produces istamycins. J. Antibiotics 34: 824~829, 1981
14) PIENDL, W.; A. BÖCK & E. CUNDLIFFE: Involvement of 16S ribosomal RNA in resistance of the aminoglycoside-producers *Streptomyces tenjimariensis*, *Streptomyces tenebrarius* and *Micromonospora purpurea*. Mol. Gen. Genet. 197: 24~29, 1984
15) SATOH, A.; H. OGAWA & Y. SATOMURA: Effect of sclerin on production of the aminoglycoside antibiotics accompaired by salvage function in *Streptomyces*. Agric. Biol. Chem. 39: 1593~1598, 1975
16) NAKANO, M. M.; H. MASHIKO & H. OGAWARA: Cloning of the kanamycin-producing gene from a kanamycin-producing *Streptomyces* species. J. Bacteriol. 157: 79~83, 1983
17) NATHANS, D. & F. LIPMANN: Amino acid transfer from aminoacyl-ribonucleic acids to protein on ribosomes of *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. 47: 496~504, 1961
18) SUGIYAMA, M.; S.-Y. PAIK & R. NOMI: Mechanism of self-protection in a puromycin-producing microorganism. J. Gen. Microbiol. 131: 1999~2005, 1985
19) PÉREZ-GONZÁLEZ, J. A.; J. VARA & A. JIMÉNEZ: Acetylation of puromycin by *Streptomyces alboniger*, the producing organism. Biochem. Biophys. Res. Commun. 113: 772~777, 1983
20) PAIK, S.-Y.; M. SUGIYAMA & R. NOMI: Isolation and properties of a puromycin acetyltransferase from puromycin-producing *Streptomyces alboniger*. J. Antibiotics 38: 1761~1766, 1985
21) YAMAGUCHI, H.; C. YAMAMOTO & N. TANAKA: Inhibition of protein synthesis by blasticidin S. I. Studies with cell-free systems from bacterial and mammalian cells. J. Biochem. 57: 667~677, 1965
22) YAMAGUCHI, H. & N. TANAKA: Inhibition of protein synthesis by blasticidin S. II. Studies on the site of action in *E. coli* polypeptide synthesizing systems. J. Biochem. 60: 632~642, 1966
23) KEERATIPIBUL, S.; M. SUGIYAMA, O. NIMI & R. NOMI: Streptothricin production by a new isolate of *Streptomyces* from Thailand soil. J. Ferment. Technol. 62: 19~28, 1984
24) KEERATIPIBUL, S.; M. SUGIYAMA & R. NOMI: Mechanism of resistance to streptothricin of a producing microorganism. Biotechnol. Lett. 5: 441~446, 1983
25) THOMPSON, C. J.; J. M. WARD & D. A. HOPWOOD: DNA cloning in *Streptomyces*: resistance genes from antibiotic-producing species. Nature 286: 525~527, 1980
26) SHAW, W. V. & D. A. HOPWOOD: Chloramphenicol acetylation in *Streptomyces*. J. Gen. Microbiol. 94: 159~166, 1976
27) SINKAWA, H.; M. SUGIYAMA, O. NIMI & R. NOMI: Molecular cloning and expression in *Streptomyces lividans* of a streptomycin 6-phosphotransferase gene from a streptomycin-producing microorganism. FEBS Lett. 181: 385~389, 1985