INDUCTION OF STREPTOMYCIN-INACTIVATING ENZYME BY A-FACTOR IN *STREPTOMYCES GRISEUS*

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The effect of A-factor on streptomycin resistance and productivity in *Streptomyces griseus* and *S. bikiniensis* was studied using A-factor-negative mutants. Resistance of several of these mutants was markedly increased by adding A-factor to the growing medium, as also was their streptomycin productivity. The A-factor induced resistance was due to inactivation by streptomycin-6-phosphotransferase, and enzyme synthesis in these mutants was completely dependent on the presence of A-factor. In the case of *S. griseus* 2247 where streptomycin productivity was independent of A-factor, resistance and synthesis of the inactivating enzyme were also independent of A-factor. A-Factor-negative mutants of *S. griseus* showed a decreased level of NADP-glycohydrolase and an increased level of several NADP-linked dehydrogenases, but these enzymes did not return to parental levels in cultures supplemented with A-factor. A-Factor seems to regulate streptomycin biosynthesis, not through an indirect metabolic sequence involving these enzymes but, more likely, by directly stimulating synthesis of enzyme(s) in the biosynthetic pathway.

A-Factor (2*S*-isocapryloyl-3*S*-hydroxymethyl-γ-butyrolactone) was first reported by KHOKHLOV *et al.*[1~4] as a stimulatory factor for both streptomycin biosynthesis and spore formation in *Streptomyces griseus* and *S. bikiniensis*. We recently found that streptomycin-non-producing mutants derived from *S. griseus* FT-1 by UV-irradiation could be classified into major two classes, one blocked in the pathway of streptomycin biosynthesis and the other deficient in the synthesis of A-factor.[5] The A-factor-negative mutants were restored to streptomycin productivity when A-factor was added to the growth medium at very low concentration. We also observed that similar streptomycin-nonproducing mutants deficient for A-factor were obtained with high frequency by treatment with intercalating dyes or incubation at high temperature. It was suggested that a plasmid or transposable element controlled the biosynthesis of streptomycin through A-factor.

A close relationship between streptomycin resistance and productivity has been observed with antibiotic-producing strains. SHAW and PIWOWARSKI[6,7] reported that treatment of *S. bikiniensis* with dyes resulted in loss of streptomycin resistance as well as loss of streptomycin productivity with high frequency. They observed that cell-free extracts of the dye-treated isolates did not inactivate streptomycin due to the absence of streptomycin-6-phosphotransferase activity. KIRBY and LEWIS[8] also described asporogenic mutants of *S. griseus* and *S. bikiniensis* concomitantly lacking of streptomycin resistance and productivity and they suggested involvement of transposable genetic elements in this mutation.

The apparent resemblance between genetic control of streptomycin productivity and that of resistance led us to examine the effect of A-factor on the streptomycin resistance of *S. griseus*. Also

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we examined the possible effect of A-factor on NADP-glycohydrolase and NADP-linked glucose-6-phosphate dehydrogenase which were considered by VORONINA *et al.*[9] to be key reactions in the mechanism by which A-factor regulates streptomycin biosynthesis. In this report we describe induction of streptomycin resistance by A-factor through an increase in streptomycin-6-phosphotransferase. The mechanism of the stimulatory effect of A-factor on streptomycin biosynthesis is also discussed.

Materials and Methods

Microorganisms

Streptomyces griseus FT-1 was obtained from Meiji Seika Kaisha, Ltd., Tokyo. *S. griseus* IFO 13189 and *S. bikiniensis* IFO 13350 were obtained from the Institute for Fermentation, Osaka. *S. griseus* 2247 was provided by R. NOMI, Hiroshima University. As reported previously,[5] this strain does not require A-factor for streptomycin biosynthesis. The A-factor-negative mutant of *S. griseus* FT-1 was derived by UV-irradiation; those of *S. griseus* IFO 13189 and 2247 were obtained by acridine orange treatment and that of *S. bikiniensis* by incubation at high temperature. *Bacillus subtilis* ATCC 6633 was used to bioassay streptomycin.

Medium

The liquid medium to produce streptomycin and test for streptomycin resistance was glucose - meat extract - peptone (GMP) medium containing 1% glucose, 0.2% yeast extract, 0.2% meat extract, 0.4% peptone, 0.5% NaCl and 0.025% $MgSO_4 \cdot 7H_2O$, pH 7.0.[10]

Materials

Synthetic A-factor [(3*S*)-(−)-A-factor] was provided by K. MORI[11] of this department. Streptomycin, streptidine and *O*-α-L-dihydrostreptose(1→4)streptidine were obtained from Meiji Seika Kaisha, Ltd. *Escherichia coli* alkaline phosphatase, adenosine 5′-triphosphate disodium salt (ATP), glucose-6-phosphate, isocitrate and 6-phosphogluconate were purchased from Sigma Chemical Company. Nicotinamideadenine dinucleotide phosphate (NADP) was purchased from Oriental Yeast Co., Ltd., Osaka.

Streptomycin Resistance

Cultures of each strain in 10 ml of GMP medium incubated in test tubes (21 mm diameter) for 2 days at 26.5°C were transferred at a dilution of 1: 200 into 10 ml of the fresh medium containing various concentrations of streptomycin with or without A-factor. Growth was observed after further incubation for 3 days at 26.5°C.

Assay of Streptomycin-inactivating Enzyme

S. griseus and *S. bikiniensis* were grown in GMP medium. For the preparation of cell-free extracts and reaction mixture, the procedure described by PIWOWARSKI and SHAW[7] was used. Cells were collected and washed with TMS buffer (10 mM tris-HCl, 10 mM magnesium chloride, 25 mM ammonium chloride, 0.6 mM 2-mercaptoethanol, pH 7.8). Washed cells were resuspended in TMS buffer and sonically disrupted. Sonicated cell suspensions were centrifuged at 25,000×*g* for 20～30 minutes, and the supernatants were used as enzyme solutions. Protein was determined by the colorimetric assay of LOWRY *et al.*[12] Assay mixtures containing equal volumes of cell-free extract, 1 mM streptomycin sulfate, 40 mM ATP (in water, pH 7.6) and 100 mM magnesium acetate were incubated for 1 hour at 30°C. As phosphorylated streptomycin is biologically inactive, the amount formed was measured by the decrease of antibiotic activity in the reaction mixture. Streptomycin was assayed by the paper disc method.

Streptomycin-phosphate was dephosphorylated by phosphatase. Assay mixtures containing equal volumes of the reaction mixture in which enzyme activity had been stopped by heat treatment, 50 mM tris-HCl, pH 9.0, and an adequate volume of *E. coli* alkaline phosphatase were incubated for 1 hour at 37°C.

Identification of Compounds

Streptomycin, streptidine, *O*-α-L-dihydrostreptose(1→4)streptidine and their phosphorylated derivatives were identified by high performance liquid chromatography.[5]

Assay of NADP-glycohydrolase, Glucose-6-phosphate Dehydrogenase, Isocitrate Dehydrogenase and 6-Phosphogluconate Dehydrogenase

Cell-free extracts were prepared as described above, using 50 mM tris-HCl buffer, pH 7.2, substituting for TMS buffer. NADP-glycohydrolase (EC 3.2.2.6) was measured according to VORONINA *et al.*[9] Each assay mixture contained 0.3 ml of 2 mM NADP and approximately 100 μg of extracted mycelial protein. After incubation at 37°C, 2 ml of 1 M potassium cyanide was added to 0.1 ml of the reaction mixture and formation of the cyanide complex of intact NADP was measured at 325 nm.

NADP-linked dehydrogenases were assayed at 25°C by following the increase in absorbance of NADPH at 340 nm. The reaction mixtures described by GRÄFE *et al.*[18] were used. The assay mixture for glucose-6-phosphate dehydrogenase (EC 1.1.1.49) contained, in 3.0 ml: 2.6 ml of 100 mM triethanolamine buffer, pH 7.6, 0.1 ml of 30 mM glucose-6-phosphate, 0.1 ml of 200 mM magnesium chloride, 0.1 ml of 12 mM NADP, and 0.1 ml of cell-free extracts. The assay mixture for isocitrate dehydrogenase (EC 1.1.1.42) contained, in 3.0 ml: 2.6 ml of 100 mM triethanolamine buffer, pH 7.5, 0.1 ml of 150 mM isocitrate, 0.1 ml of 120 mM manganese sulfate, 0.1 ml of 30 mM NADP, and 0.1 ml of cell-free extracts. The assay mixture for 6-phosphogluconate dehydrogenase (EC 1.1.1.44) contained, in 3.0 ml: 2.6 ml of 100 mM triethanolamine buffer, pH 7.4, 0.1 ml of 60 mM 6-phosphogluconate, 0.1 ml of 300 mM magnesium sulfate, 0.1 ml of 30 mM NADP, and 0.1 ml of cell-free extracts.

One unit of these enzymes were expressed by amounts (nmoles) of the substrate decreased or the products increased per minute.

Results

Effect of A-Factor on Streptomycin Resistance

Table 1 shows the maximum concentrations of streptomycin permitting growth of various strains of *S. griseus* and *S. bikiniensis* in the presence and absence of A-factor in the medium. The resistance of *S. griseus* FT-1 is remarkably high and matches its high streptomycin productivity. An A-factor-negative mutant of this strain grown in the absence of A-factor was only one tenth as resistant. However a supplement of A-factor caused complete recovery to the level of the parental FT-1. Other A-

Table 1. Streptomycin resistance of various strains of *S. griseus* and *S. bikiniensis* and effect of A-factor.

Strains	A-Factor added to medium	Streptomycin resistance (μg/ml)	Streptomycin production
S. griseus FT-1 (A-factor$^+$)	—	>10,000	+
S. griseus FT-1 (A-factor$^-$)	—	1,200	−
S. griseus FT-1 (A-factor$^-$)	1 μg/ml	>10,000	+
S. griseus IFO 13189 (A-factor$^+$)	—	100	+
S. griseus IFO 13189 (A-factor$^-$)	—	10	−
S. griseus IFO 13189 (A-factor$^-$)	1 μg/ml	40	+
S. griseus 2247 (A-factor$^+$)	—	500	+
S. griseus 2247 (A-factor$^-$)	—	250	+
S. griseus 2247 (A-factor$^-$)	1 μg/ml	250	+
S. bikiniensis IFO 13350 (A-factor$^+$)	—	80	+
S. bikiniensis IFO 13350 (A-factor$^-$)	—	40	−
S. bikiniensis IFO 13350 (A-factor$^-$)	1 μg/ml	60	+

factor-negative mutants derived from FT-1 treatment with acridine orange or by incubation at high temperature gave similar results. With the A-factor-negative mutants of *S. griseus* IFO 13189 and *S. bikiniensis* IFO 13350, the response to exogeneously added A-factor was not as complete but a distinct recovery was observed.

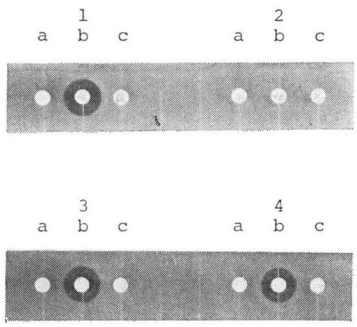
In contrast, the A-factor-negative mutant of *S. griseus* 2247 was different. Although it had only half the resistance of its parent, it did not respond to the addition of A-factor. As reported in a previous paper,[5] the streptomycin productivity of this strain was also independent of A-factor.

Effect of A-Factor on Streptomycin-phosphorylating Enzyme Activity

It is well known that at least part of the streptomycin resistance of the antibiotic-producing strains is due to inactivation of the antibiotic by a streptomycin-phosphorylating enzyme. Therefore, the effect of A-factor on the activity of the enzyme was examined with cell-free extracts of various strains grown in the presence and absence of A-factor. When streptomycin was incubated with cell-free extracts of all the parental strains in the presence of ATP and Mg^{2+}, complete inactivation of the antibiotic was observed (Fig. 1). Inactivating activity was absent from cell-free extracts of the A-factor-negative mutants of *S. griseus* FT-1, IFO 13189 and *S. bikiniensis* IFO 13350, but it was unambiguously present when these mutants were grown in the presence of A-factor. Similar dependence of inactivating activity on A-factor was also observed with a double mutant of *S. griseus* FT-1 (class III mutant previously reported[5]) which had a defect in streptomycin biosynthetic pathway in addition to A-factor deficiency (data not shown). The mutant of *S. griseus* 2247 possessed inactivating activity, irrespective of A-factor supplementation.

Fig. 1. Streptomycin inactivation by cell-free extracts.

1, *S. griseus* FT-1. 2, *S. griseus* 2247. 3, *S. griseus* IFO 13189. 4, *S. bikiniensis* IFO 13350. a, parent. b, A-factor-negative mutant grown without A-factor. c, A-factor-negative mutant grown with A-factor.



Products of the streptomycin-inactivating enzyme reaction were analyzed by high performance liquid chromatography (Fig. 2). When inactivation occurred during incubation with cell-free extracts of *S. griseus* FT-1 in the presence of ATP, the streptomycin peak disappeared and a new peak, probably streptomycin-phosphate, was clearly observed. No such change was observed in the absence of ATP or cell-free extracts. The product was converted to streptomycin by treatment with *E. coli* alkaline phosphatase. Moreover, streptidine and *O*-α-L-dihydrostreptose(1→4)streptidine were also phosphorylated by these cell-free extracts to the corresponding phosphorylated substances which were converted to the original substrates by treatment with alkaline phosphatase.

From these results, we conclude that the streptomycin-inactivating enzyme of *S. griseus* FT-1 is streptomycin-6-phosphotransferase.

Time Course of Induction of Streptomycin-6-phosphotransferase by A-Factor

Fig. 3 shows the activity of streptomycin-6-phosphotransferase in growing cultures of *S. griseus*

Fig. 2. Detection of products of streptomycin-inactivating enzyme by high performance liquid chromatography.

Cell-free extracts from *S. griseus* FT-1 was used. Experiments 1~5 were carried out using streptomycin as substrate (1, before reaction; 2, after reaction; 3, reaction without ATP; 4, reaction without cell-free extracts; 5, treated with *E. coli* alkaline phosphatase after the inactivation reaction). Experiments 6 and 7 with streptidine as substrate (6, after reaction; 7, treated with phosphatase). Experiments 8 and 9 with *O*-α-L-dihydrostreptose(1→4)streptidine as substrate (8, after reaction; 9, treated with phosphatase). 10, control (a, streptomycin; b, *O*-α-L-dihydrostreptose (1→4)streptidine; c, streptidine).

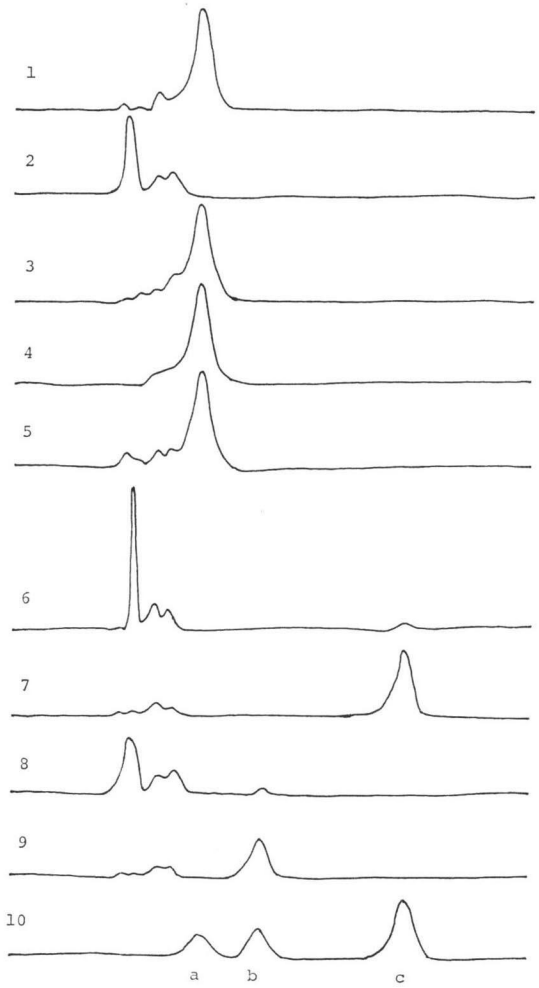
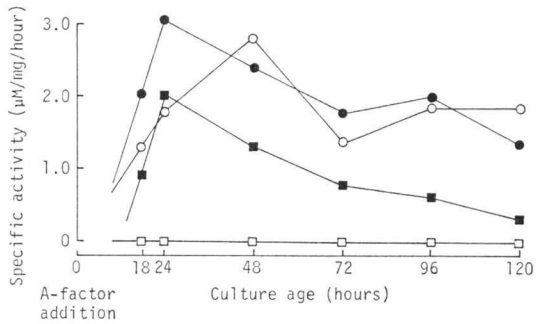


Fig. 3. Effect of A-factor supplements on the activity of streptomycin-phosphorylating enzyme in cultures of *S. griseus* FT-1 and its A-factor-negative mutant.

Cells collected from a seed culture grown in GMP medium at 28°C for 2 days, were washed once by centrifugation to remove A-factor and suspended in fresh GMP medium of the same volume. A 1-ml suspension was used to inoculate 100 ml of GMP medium (glucose, 3%) in a 500-ml Sakaguchi flask. Cultivation was carried out at 28°C on a reciprocal shaker.

(○) Parent. (●) Parent, 1 μg/ml of A-factor added to medium. (□) A-Factor-negative mutant. (■) A-Factor-negative mutant, 1 μg/ml of A-factor added to medium.



FT-1 and its A-factor-negative mutant, with or without added A-factor. When the parent strain was cultured in the medium without A-factor, the enzyme appeared soon after the inoculation and reached a maximum after 48 hours. Addition of A-factor to the medium at 0 hour caused a distinct stimulation of enzyme synthesis and maximum activity was reached at 24 hours. In the A-factor-negative mutant, no enzyme activity was detected in the unsupplemented culture whereas appreciable enzyme synthesis was observed when A-factor was present. In the case of the A-factor independent strains, *S. griseus* 2247 and its A-factor-negative mutant, addition of A-factor caused no stimulative effect.

Measurement of NADP-glycohydrolase and Several NADP-linked Dehydrogenases

As shown in Table 2, parental strains of *S. griseus* FT-1, IFO 13189 and 2247 have relatively high

Table 2. Activities of NADP-glycohydrolase and several NADP-linked dehydrogenases of *S. griseus* FT-1, IFO 13189, 2247 and their A-factor negative mutants.

Strains	A-Factor added to medium	Specific activities (units/mg protein)											
		NADP-glycohydrolase			Glucose-6-phosphate dehydrogenase			Isocitrate dehydrogenase			6-Phosphogluconate dehydrogenase		
		24 hours	48 hours	72 hours	24 hours	48 hours	72 hours	24 hours	48 hours	72 hours	24 hours	48 hours	72 hours
FT-1 (A-factor$^+$)	—	1,300	4,400	5,600	18	0	0	330	85	25	0	0	0
FT-1 (A-factor$^-$)	—	130	460	210	34	31	20	810	570	470	8.3	7.2	2.4
FT-1 (A-factor$^-$)	1 μg/ml	120	100	90	32	24	19	660	430	370	6.1	6.1	2.4
IFO 13189 (A-factor$^+$)	—	2,800	1,200	3,000	6	6	0	200	160	0	0	0	0
IFO 13189 (A-factor$^-$)	—	250	120	170	22	29	10	310	430	200	1.2	4.6	0.6
IFO 13189 (A-factor$^-$)	1 μg/ml	100	50	20	16	13	13	180	140	190	0	0	0.5
2247 (A-factor$^+$)	—	1,900	5,300	7,400	0	0	0	240	52	0	0	0	0
2247 (A-factor$^-$)	—	130	190	200	25	21	15	340	280	210	0	1.3	0
2247 (A-factor$^-$)	1 μg/ml	80	130	50	23	21	16	280	290	220	0	0.7	0

NADP-glycohydrolase activity, while their A-factor-negative mutants, even that of strain 2247, have a very low level of this enzyme. Conversely, the activities of several NADP-linked dehydrogenases *i.e.*, glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase and 6-phosphogluconate dehydrogenase, were absent or very weak in the parent strains but high in all A-factor-negative mutants. The enzyme levels in these mutants did not return to the parental level upon addition of A-factor to the cultures.

The marked difference in activity between the NADP-linked dehydrogenases of the parents and those of the A-factor-negative mutants was not observed when the assay mixtures contained 10-times more NADP. This suggests that cell-free extracts of the parental strains contain higher concentrations of a competitive inhibitor of NADP, probably phosphoadenosine diphosphoribose as suggested by VORONINA *et al.*[9]

Discussion

The results in this paper show that streptomycin-6-phosphotransferase-mediated streptomycin resistance in *S. griseus* is induced by A-factor. Induction of the inactivating enzyme by A-factor occurred in the mutant having a defect in streptomycin biosynthetic pathway. This excludes a possibility that A-factor induces the enzyme synthesis indirectly followed by the activation of streptomycin production. The increase in streptomycin resistance brought about by A-factor in the negative mutants of *S. griseus* IFO 13189 and *S. bikiniensis* IFO 13350 was not as complete as observed in the mutant of *S. griseus* FT-1. However, synthesis of the inactivating enzyme depended completely on A-factor, in all these strains. It is well known that streptomycin resistance in antibiotic-producing strains is due to the presence of both the inactivating enzyme and a cell membrane barrier preventing penetration of the antibiotic. It seems reasonable to assume that the elevated resistance of *S. griseus* FT-1 was largely due to an increase in the inactivating enzyme level. Because of this the response to A-factor is enhanced. Considering also our earlier results,[5] it is now evident that an unstable genetic determinant for A-factor biosynthesis controls streptomycin resistance as well as streptomycin productivity and spore formation in this organism. Control is exerted through the regulatory effect of A-factor. Mutants lacking streptomycin productivity as well as resistance were reported by SHAW and PIWOWARSKI[6,7] and KIRBY and LEWIS.[8] It seems highly probable that their mutants were, in fact, A-factor deficient.

VORONINA *et al.*[9] proposed a mechanism for A-factor stimulation of streptomycin productivity whereby A-factor primarily stimulated NADP-glycohydrolase. This would increase the amount of phosphoadenosine diphosphoribose, which in turn would inhibit NADP-linked glucose-6-phosphate dehydrogenase and the pentose phosphate pathway. They assumed that glucose was preferentially used for streptomycin biosynthesis under such metabolic condition. We could confirm their observation that A-factor-negative mutants had lower NADP-glycohydrolase activity and higher NADP-linked dehydrogenases activities, probably due to the decreased level of phosphoadenosine diphosphoribose. However, we could not observe recovery of parental enzyme levels in mutants to which A-factor was added. This suggests that A-factor is not a regulatory effector for these NADP-related enzymes, even though it evidently regulates the level of streptomycin-6-phosphotransferase. In most of the A-factor-negative mutants tested, streptomycin production was restored by adding A-factor without significantly altering the level of the NADP-relating enzymes. Streptomycin productivity in the A-factor-negative mutant from strain 2247 was not affected by A-factor deficiency but it still had abnormal enzyme levels. These results indicate that there are no direct links between streptomycin biosynthesis and the activities of NADP-glycohydrolase and glucose-6-phosphate dehydrogenase. A-Factor may affect streptomycin biosynthesis more directly by causing an increased level of enzyme(s) in the biosynthetic pathway as it does with streptomycin-6-phosphotransferase.

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