Inhibition of Spore Formation and Thiostrepton Production by Accumulated Intermediates of Purine Biosynthesis and the Suppression by AICA (5Amino-4-Imidazole Carboxamide) in Purine Auxotrophic Mutants of Streptomyces azureus ATCC 14921

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Inhibition of Spore Formation and Thiostrepton Production by Accumulated Intermediates of Purine Biosynthesis and the Suppression by AICA (5-Amino-4-Imidazole Carboxamide) in Purine Auxotrophic Mutants of *Streptomyces azureus* ATCC 14921

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The two types of purine auxotrophs of *Streptomyces azureus* required adenine and other purines at less than 50 μg/ml for their growth, but their spore formation was inhibited by excess adenine and hypoxanthine. An excess of these purines stimulated a remarkable accumulation of intermediates (especially AICAR, 5'-phosphoribosyl-5-amino-4-imidazole carboxamide) of purine biosynthesis in the mutants. The inhibitory effect of excess purines on spore formation was proportional to the amount of intermediates accumulated. No such inhibition and intermediate accumulation occurred with guanine and xanthine and with the wild-type strain. An adenine related compound AICA (5-amino-4-imidazole carboxamide) suppressed the inhibitory effect of the purines and bypassed the accumulation of the intermediates. The mutants produced very low levels of the antibiotic thiostrepton. AICA also stimulated the thiostrepton production of mutants as well as their growth. AICA enhances the mycelial metabolism, and thus the accumulation of intermediates, which suppresses spore formation and thiostrepton production, may be prevented in the mutants.

INTRODUCTION

Streptomyces species differentiate from substrate mycelia to aerial mycelia and finally produce with spores with species- (or strain-) specific color. They produce numerous kinds of antibiotics by secondary metabolism. The antibiotic production is known to be link to the differentiation. The factors or substances effecting the two processes have been known in some *Streptomyces* spp. (Ochi, 1984). The study of these factors is especially of interest because they should offer a key to reveal the relationship between the two processes. However, little is known about nucleic acid related compounds as effecting factors, except GTP, ppGpp (guanoshine 5'-diphosphate 3'-diphosphate) and some others (Ochi, 1984, 1986, 1987).

We have isolated two kinds of artificially induced purine auxotrophs in the thiostrepton-producing strain *Streptomyces azureus* ATCC 14921 : 1) mutant Ade2 with purple spores and its derivative Ade21 with yellowish orange spores which accumulat-

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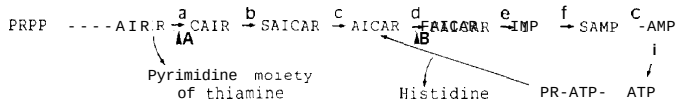

Fig. 1. Step blocked on purine synthetic pathway in purine auxotrophic mutants ATH, Ade2 and Ade21 of *Streptomyces azureus*.

A, blocked step in mutants Ade2 and Ade21; B, blocked step in mutant ATH. CAIR, 5'-phosphoribosyl5-aminoimidazole-4-carboxylic acid ; SAICAR, [5'-phosphoribisyl 4-(N-succinocarboxamide)-5-aminoimidazole]; SAMP, adenylosuccinic acid.

ed AIR (5'-phosphoribosyl-5-aminoimidazole) due to the lack of AIR carboxylase activity (Yamada et *al., 1990)* ; 2) mutant ATH with bluish green spores like those of the wild-type strain and which accumulated AICAR due to the lack of AICAR formyltransferase activity (Ogata and Yamada, 1990), as shown in Fig. 1. The mutants Ade2 and ATH required adenine and other purines at less than 50 μg/ml for their growth, whereas their spore formation was inhbited by excess adenine and hypoxanthine (Ogata and Yamada, 1990). The inhibition seemed to be due to the purine auxotrophy. An addition of AICA suppressed the inhibitory effect of excess adenine and hypoxanthine.

Freese and co-worker had reported a similar inhibition of spore formation by excess adenine and its analogues or inhibitors of purine biosynthesis on *Bacillus subtilis* and its purine auxotrophs (Abedine et al., 1983 ; Heinze *et al.*, 1978). However, they had not clarified the mechanisms of inhinition, and had not reported the suppression of inhibition.

The mutants Ade2 and ATH produced very low levels of the antibiotic thiostrepton. This character also seemed to be due to the purine auxotrophy. This paper describes the cause of the inhibition of spore formation by excees purines, the AICA suppression of the inhibition of spore formation and the blocked antibiotic production and the AICA stimulation of thiostrepton production by the purine auxotrophs. This phenomena is a novel observation in streptomycetes. Furthermore, studies on the purine auxotrophs have rarely been conducted for the streptomycetes.

MATERIALS AND METHODS

Organisms, media and cultural conditions. *Streptomyces azureus* ATTC 14921 (wild-type strain PKO) and its purine auxotrophic mutants were used in this work and cultivated in rye flake agar medium, Bennett agar and broth media and minimal medium under the conditions reported previously by Ogata *et al.*, (1985a), Ogata and Yamada (1990) and Yamada et *al.* (1990).

Inhibition of spore formation. The inhibition of spore formation was examined as previously described by Ogata and Yamada (1990). The spores of each strain were spread on the surface of Bennett agar plates on which paper disks (8 mm in diameter ; each containing 200 μg purine) were then placed and incubated at 28°C for 7 days. The inhibitory zones around the paper disks were measured.

Thiostrepton productivity. Thiostrepton productivity of these mutants were determined as previously described by Ogata et *al.*, (1985a, 198513). Thiostrepton production in the liquid was determined using the Bennett broth supplemented with 1 % corn starch. The mycelia grown for 4 days were gathered by centrifugation at 3,000 xg for 10 min and then applied to DMSO-extraction. Thiostrepton activity in the extract was determined by the paper disk method with *Bacillus subtilis* ATCC 6633 as test organism.

The thiostrepton productivity in the plate culture was also determined as described by Ogata et al., (1985b)

Analysis of intermediates in the purine synthetic pathway. The accumulation of intermediates such as AIR, AICAR and others in the purine biosynthetic pathway of the mutants was determined according to the previous papers (Ogata and Yamada, 1990 ; Yamada et *al.*, 1990). Minimal medium supplemented with 10 μg/ml adenine (for mutants Ade2 and Ade21)or 10 μg/ml adenine and 1 μg/ml thiamine (for mutant ATH) was used for this experiment as a basal medium. The mycelia from 2 day's culture in Bennett broth was washed twice with the basal medium, and then incubated for 24 hr in the basal medium with or without excess purines (200 μg/ml). A 5- mg (wet weight) portion of the mycelia was extracted with 10 ml of 1 N folic acid for 30 min in an ice bath. The amount of AIR, AICAR or others in each extract was determined, according to the Bratton-Marshall test (Ogata and Yamada, 1990 ; Yamada et al., 1990).

RESULTS

Effect of excess purines on spore formation

The spore formation of mutants ATH and Ade2 was clearly inhibited by excess adenine and hypoxanthine (Fig. 2), but the inhibitory effect of hypoxanthine was fairly less than that of adenine, as shown in Table 1. A very weak inhibition of spore formation by adenine was also observed on mutant Ade21, but not by hypoxanthine. AICA (SIGMA Chemical Co.) at 10μg/ml suppressed the inhibitory effect of adenine

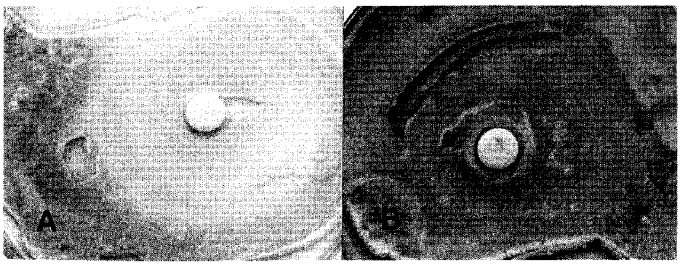

Fig. 2. Inhibition of spore formation of mutant ATH by excess adenine.

A, Mutant strain ATH ; B, Wild-type strain PKO. Bennett agar medium was used in this experiment, and a paper disc (8 mm in diameter) contained 200 μg. The same inhibition of spore formation was observed on mutant Ade2

Table 1. Effect of excess purines on the spore formation of the wild-type strain PKO and its purine auxotrophic mutants in *Streptomyces azureus*.

Purine	strains PKO	ATH	Ade2	Ade21
Control				
Adenine		+ + +	+ + +	(+)
Guanine			−	
Hypoxanthine		+ +	+	
Xanthine			−	
Adenine (+ AICA)				
Hypoxanthine (+ AICA)			−	

+ + +, Inhibitory zone was greater than 2 cm in diameter ; + +, 1 to 2 cm ; +, less than 1 cm ; (+), less than 1 cm but spore color appeared in the inhibitory zone during further cultivation ; −, no effect. The experiments were performed as follows. The spores were spread on the surface of Bennett agar plates, and paper disks (8 mm in diameter ; each containing 200 μg purine) were put on top. AICA (10 μg/ml) was added to the agar medium. The plates were incubated at 28°C for 3 days, and the inhibitory zones around the paper disks were measured.

and hypoxanthine on these mutants. No such inhibition occurred with guanine and xanthine and with the wild-type strain PKO. Similar inhibition by excess adenine and its analogues has been known in the purine auxotrophs of *B. subtilis* (Abedin et al., 1983 ; Freese *et al., 1979).* It has been also reported that spore formation and cell growth of a purine auxotroph of *B. subtilis* were stimulated by a small amount of purines added (Heinze et *al., 1978).* But, the mechanisms or the mode of action have been clarified.

Abnormal accumulation of AICAK or AIR in the presence of excess purines

Table 2 shows the accumulation of the intermediates in the purine synthetic pathway of the wild-type strain PKO and its mutants with or without excess purines. ATH accumulated AICAR and Ade2 or Ade21 accumulated AIR. PKO showed no

Table 2. Accumulation of purine intermediates in purine auxotrophic mutants of *Streptomyces azureus* in the presence of excess purines.

Purine	Accumulation (/*g/ml) Strains PKO	ATH	Ade2	Ade21
Control	ND	AICAR (0.1)	AIR (0.2)	AIR (0.1)
Adenine	ND	AICAR (5.2)	AICAR (20.0)	AICAR (8.0)
Guanine	ND	AICAR (0.5)	AICAR (1.0)	AICAR (0.6)
Hypoxanthine	ND	AICAR (2.4)	AIR (2.5)	AIR (0.7)
Xanthine	ND	AICAR (0.3)	AIR (0.4)	AIR (0.6)

ND, not detectable; PKO, Wild-type strain.
The numbers in the parentheses show the actual values of accumulated AICAR or AIR. Purines (200 μg/ml) were added to the basal medium.

accumulation of intermediates. An excess of adenine increased the accumulation of AICAR in mutant ATH, and also induced a remarkable accumulation of AICAR in mutants Ade2 and Ade21, but the latters did not accumulate AICAR in the control without excess adenine. Excess hypoxanthine increased accumulation of AICAR in ATH, but not in Ade2 and Ade21. In Ade2, hypoxanthine enhanced a pronounced accumulation of AIR. The inhibitory effect of excess adenine and hypoxanthine on spore formation was proportional to the amount of accumulated AICAR or AIR. However, the inhibitory effect of hypoxanthine on Ade2 was somewhat less than that of adenine on the mutants ATH and Ade2 or of hypoxanthine on ATH (Tables 1 and 2). Guanine and xanthine, which had no inhibitory effect on the spore formation, hardly had any significant effect on the accumulation of AICAR or AIR in the mutants.

The correlation between AICAR accumulation and depressed spore formation suggests that AICAR itself may be directly responsible for the effect. The pronounced accumulation of AIR also appears to inhibit the spore formation of Ade2. In the presence of AICA (10 μg/ml), excess hypoxanthine did not enhance the amount of AIR (data not shown). The amount of AICAR seemed to decrease in the presence of AICA, but AICA interferes with measurement of AICAR. The AICA suppression of the inhibition of spore formation by excess purines is likely due to the decrease in production of AICAR or AIR. We propose that an excess of adenine and hypoxanthine affects the purine biosynthetic pathway in the mutants, so that it brings about an abnormal accumulation of AICAR or AIR, causing a harmful effect on the spore formation of the mutants. However, there would be some difference between the mechanisms of AICAR accumulation in ATH and Ade2 or Ade21.

Effect of AICA on thiostrepton production and mycelial growth

As shown in Table 3, thiostrpton produced by mutants ATH and Ade2 was barely

Table 3. Thiostrepton productivity and mycelial growth of purine auxotrophic mutants of *Streptomyces azureus* in the liquid culture.

Strain	Amount of mycelium (w. w. mg/ml)	Relative amount of mycelium (%)	Thiostrepton production (μg/w. w. mg)	Relative thiostrepton productivity (%)
Control				
PKO	44.8	100	3.0	100
ATH	44.5	99	<0.1	
Ade2	44.3	99	0.1<	3<
Ade21	44.8	100	0.3	10
AICA (10 μg/ml)				
PKO	70.5	157	4.2	140
ATH	60.1	133	<0.1	
Ade2	65.2	136	1.7	57
Ade21	67.2	150	2.3	77

PKO, wild-type strain; w. w. mg, wet weight mg of mycelia.
Thiostrepton activity in the DMSO-extract of culture of each strain was determined by the paper disk method with *Bacillus subtilis* as test organism.

Table 4. Thiostrepton productivity of purine auxotrophic mutants of *Streptomyces azureus* in the plate culture.

Strains	Thiostrepton production (μg/agar plug)	Relative productivity (%)
Control		
PKO	6.4	100
ATH	<0.1	
Ade2	2.0	31
Ade21	4.1	64
AICA (10 μg/ml)		
PKO	9.2	143
ATH	<0.1	
Ade2	3.4	53
Ade21	5.9	92

PKO, wild-type strain.
The thiostrepton activity of each strain grown on the agar plate was determined by the agar piece (plug) method with ***Bacillus*** *subtilis* as test organism. A plug was 8 mm in diameter and 5 mm in thickness.

detected in the liquid culture. That of mutant Ade21 showed a decrease of about 90 %, compared with that of wild-type strain PKO. However, there was a significant increase in thiostrepton production of Ade2 and Ade21 as well as PKO in the presence of AICA, compared with the control without AICA. In ATH, AICA stimulation of thiostrepton production was not observed. We are still continuing to clarify the relationship between the Ath auxotrophy and blocked thiostrepton production. The same results were obtained in the solid (plate) cultures, as shown in Table 4.

AICA also enhanced the growth of the three mutants as well as PKO in the liquid culture (Table 3). Therefore, the AICA stimulation of thiostrepton production is due to the enhancement in the growth (and metabolism) of mycelia. So, none or reduced production of thiostrepton in mutants Ade2 and Ade21 (and probably ATH) may be due to their purine auxotrophy, namely the accumulation of AIR or AICAR.

Effect of exogenous AICAR on spore formation, mycelial growth and thiostrepton production

To confirm the inhibitory effect of AICAR on the spore formation, mycelial growth and thiostrepton production, exogenous AICAR (SIGMA Chemical Co.) was added to the cultures at 10 μg/ml. Exogenous AICAR showed no significant effect (or perhaps a slight stimulation) of the spore formation, mycelial growth and thiostrepton production (data not shown). This result could be due to the lack of penetration of AICAR into the cells or its decomposition into AICA and others outside of them.

DISCUSSION

We suppose that an excess of adenine and hypoxanthine affects purine biosynthetic pathway in the mutants, so that they bring about an abnormal accumulation of AICAR or AIR and subsequently a harmful effect on their spore formation. A

significant accumulation of AICAR and AIR is also considered to have a harmful effect on the thiostrepton production of mutants.

Furthermore, we suppose that AICA suppression on the inhibition of spore formation by excess adenine and hypoxanthine would be due to the same reason as AICA stimulation of thiostrepton production. AICA enhances mycelial metabolism, and thus the accumulation of AICAR or AIR, which suppresses spore formation, may be prevented in the mutants. However, the relationship between the Ath auxotrophy and blocked thiostrepton production remained to be resolved.

We are continuing to clarify the mechanisms of accumulation of AICAR or AIR and their mode of action on the blocked production of spores and thiostrepton. Further study is doing to clarify the different sensitivity of Ade2 and its derivative Ade21 to excess purines and the stronger effect of adenine than that of hypoxanthine on the accumulation of intermediates of purines.

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