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Stimulation of Spore Formation, Mycelial Growth and Thiostrepton Production by several Thiol Compounds and β-NAD in *Streptomyces azureus* ATCC 14921 and Its Derivatives

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Sporulation-stimulating compounds were screened by using mutant PKlOOa of *Streptomyces azureus* ATCC 14921 (wild-type strain PKO), in which spore formation was inhibited by a pock-forming plasmid pSA1.1. On agar media, L- and D-cysteines, bacitracin, glutathione, and β-NAD induced the formation of colored spores or spore mass of the strain PKlOOa. These compounds also stimulated the spore formation of strain PKO and its good spore-forming (plasmid-free) strain PK100C, and the growth of aerial and submerged mycelia of these three strains. L- and D-cysteines allowed 1.3 to 1.4-fold increase in the production of antibiotic thiostrepton in strains PKO and PK100C. The thiostrepton production of strain PKlOOa, which hardly produced it, was also increased by L- and D-cysteines by as much as 20% of that of strain PKO.

INTRODUCTION

Streptomyces species are prokaryotes that differentiate and produce spores with species- (or strain-) specific color. In the typical differentiation on agar media, the substrate (or vegetative) mycelium develops and later gives rise to an aerial mycelium, which is finally converted in part into chains of spores. The morphological differentiation, in particular, during the aerial mycelial formation is often associated with the production of antibiotics and other secondary metabolites (Chater, 1989; Hopwood, 1988). Great interests have been focused on the screening of substances which affect morphological differentiation, spore formation and aerial mycelial formation of streptomycetes, and also on their mode of action (Chater, 1989; Coleman and Ensign, 1982; Hopwood, 1988; Kondo et al., 1988; Khokholv, 1988; Maruo, 1987; Natsume et *al., 1989;* Ochi, 1990; Ochi et *al.,* 1984; Ogata, 1991; Ogata et *al.* 1985b; 1991; Vargha and Szabo, 1984). An abundant production of spores is necessary for the cultivation of industrial streptomycetes, because they are generally used as the first inoculum (starter).

Much interests are also focused on the inhibition of spore formation and aerial mycelial growth by pock-forming plasmids (conjugative sex plasmids) in *Streptomyces* spp. (Bibb *et al., 1977;* Hopwood *et al., 1973; 1984;* Kieser et al., 1982; Ogata, 1991).

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The spore formation and thiostrepton production in *Streptomyces azureus* were markedly decreased in the strains which carried a pSA1.1 (8.8 kb), a multicopy derivative of pock-forming plasmid pSA1 of the wild-type strain (Miyoshi et al., 1986; Ogata et *al.,* 1988). The parent pSA1 also injured its host (Ogata et al., 1981; 1989; Ogata, 1991) In this paper, we screened cysteine, bacitracin, glutathione and β-NAD (nicotinamide adenine dinucleotide) as restorers of spore formation inhibited by pSA1.1. We also investigated the effects of these compounds on spore formation, aerial mycelial growth, submerged mycelial growth and thiostrepton production in *S. azureus* ATCC 14921 (wild-type strain) and its derivatives, pSAl.l-carrying and plasmid-free (good spore-forming) strains, and discuss the function of these reagents.

MATERIALS AND METHODS

Bacterial strains

Streptomyces azureus ATCC 14921 (wild-type strain PKO) and its derivatives were used in this work (Miyoshi *et al.,* 1986). The wild-type strain PKO carried a pock-forming plasmid pSA1 (1 or less copy/host genome). A plasmid-free strain PKlOOC was obtained from strain PKlOO spontaneously, which carried a multicopy plasmid pSA1.1 (20 to 30 copies/host genome), a derivative of pSA1. Strain PKlOOC formed large numbers of spores. Strain PKlOOa was constructed by mating between strains PKlOO and PKlOOC, and carried plasmid pSA1.1 (20 to 30 copies/host genome), as described previously. The construction of strain PKlOOa was performed, because strain PKlOO derived the plasmid-free cells at a high frequency. Spore formation of strain PKlOOa was less than that of its parent PKlOO. Some characteristics of strains PKO, PKlOO and PKlOOC had been reported in the previous papers (Miyoshi *et al.,* 1986; Ogata *et al.,* 1981; 1988).

Media and cultural conditions

Rye flakes agar and Bennett broth and agar supplemented with or without 1% corn starch were used for the growth of organisms (Ogata *et al.,* 1981). The spores of strains PKO and PKlOOC were started as the first inoculation, as described previously. For the starter of strain PKlOOa, the submerged mycelia stocked at -30°C were used, because its spore formation was very poor. Equal concentrations of starter were used. The cultural temperature was 28°C. The liquid culture was carried out on the rotary shaker at 250 rpm in the flask with glass beads.

Measurement of number of spores

The spores were scrapped from 7-day-old slant cultures of rye flakes agar and suspended in 0.1M Tris-HCl buffer (pH 7.2). Chains of spores were broken down with a mixer or homogenizer as previously described (Ogata *et al.,* 1981). The mycelia were removed by repeated filtration through a filter paper (Toyo roshi no. 2). The number of spores was estimated by microscopic counts of ten random haemocytometer fields.

Measurement of length of aerial mycelium

The length of aerial mycelium was measured by the method of Schauer *et al.*

(1988). Lawn of mycelia grown for 5 days on Petri plates of rye flakes agar was excised and sliced from top to bottom, and then placed sideways on a cover slip. The cover slip was placed on a microscope, and the length of the mycelium was microscopically measured.

Weighing of mycelial mass in liquid culture

Precultivation was carried .out for 24h in Bennett broth without 1% corn starch , and then the culture broth was transferred (5% v/v) to a fresh broth with 1% corn starch. The mycelia grown for 5 days were centrifuged at 3,000xg for 10 min, and then the mycelial mass was weighed (wet weight).

Thiostrepton productivity

Thiostrepton productivity in the solid and liquid cultures was determined as previously described (Ogata et al., 1985a). The mycelia grown for 5 days in the liquid culture containing 1% corn starch were gathered by centrifugation and then applied to DMSO-extraction. The concentration of thiostrepton in the extract was determined by paper disk method with *Bacillus subtilis* ATCC 6633 as test organisms: paper disks of 8 mm in diameter were used and a standard curve was obtained with known concentration of thiostrepton (supplied by Asahi Kasei-Kogyo Ltd).

In the case of solid culture, plugs (8 mm in diameter) of agar were excised with a cork borer from the Bennett agar of Petri plates containing 1% corn starch and placed on the assay plates with the same test organisms. Thiostrepton production was indicated by a clear zone of inhibition around the agar plugs, and the concentrations of thiostrepton produced were determined by the comparison of the diameter of inhibition zones with those of paper disk (8 mm in diameter) with known concentrations of thiostrepton.

RESULTS

Detection of stimulators of spore formation

Strain PKlOOa which carried 20 to 30 copies of a multicopy plasmid pSA1.1, of which spore formation was strongly inhibited, was inoculated onto the surface of rye flakes agar of Petri plates as an indicator strain. Paper disks containing compounds such as amino acids, nucleic acid-related compounds, vitamins and antibiotics at 1 to 1000 μg/ml were placed on the plates. Cysteine (Sigma Chemical Co.), bacitracin (Sigma Chemical Co. and Asahi Kasei-Kogyo Ltd.), glutathione (Sigma Chemical Co.) and β-NAD (Kohjin Ltd.), which induced a faint bluish green color (indicating spore formation) around the paper disk were detected after 5 days or more cultivation.

The effects of these compounds on spore formation of strain PKlOOa, the wild-type strain PKO and the good spore-forming strain PKlOOC were tested. The maximum sporulation efficiency was obtained during 7-days cultivation of each strain. The results at the several concentration of‘each compound are shown in Table 1. The number of spores on the strain PKlOOa was increased 100-fold or more by these compounds compared with the control with no additions. They were also effective on the spore formation of the wild-type strain PKO and plasmid-cured strain PK100C, with 1.5 to 5-fold increment in the number of spores at the optimum concentration.

The effect on the plasmid pSA 1-carried strain PKO was more distinct than on the plasmid-cured strain PKlOOC. Therefore, the sporulation-stimulating effect of these compounds seems to be in proportion to be the inhibitory effect of plasmids. Bacitracin and P-NAD showed marked effects on the promotion of the spore formation in all the strains used. They also advanced the onset of coloring (maturation age) of spores or spore mass, as shown in Fig. 1. The effect of L-cysteine on spore formation was similar to that of D-cysteine.

Effect of cysteine, bacitracin, glutathione and P-NAD on aerial mycelium

The stimulatory effects of cysteine, bacitracin, glutathione and P-NAD on the formation of aerial mycelium were also observed. The length of the aerial mycelium estimated was shown in Table 2. The effect of bacitracin on the wild-type strain PKO was the most marked with about 3.5-fold stimulation. The growth of aerial mycelium was so obvious as to be recognized with a glance at the colonies or the lawn of plate culture. P-NAD also showed a strong effect on PKO with about a 1.8-fold increase. The effect of others rather resembled and slight. These results suggested that the stimulation of the aerial mycelium formation was closely correlated with the stimulation of the spore formation.

Table 1. Effect of compounds on the formation of spores in *Streptomyces azureus* ATCC 14921 (wild-type PKO) and its derivatives.

Compounds (μg/ml)		Strain		
		PKO	PKlOOa (spores/slant)	PKlOOC
No addition		1.8×10^8	4.6 × 10”	8.5×10^8
L-cys	(10)	1.9×10^8	5.4×10^5	9.0×10^8
L-cys	(50)	3.0×10^8	1.5×10^7	1.2×10^9
L-cys	(100)	2.7×10^8	1.0×10^7	9.5×10^8
D-Cys	(50)	3.0×10^8	9.8 × 10”	1.1×10^9
Bacitracin	(10)	2.8×10^8	1.0 × 10’	1.0×10^9
Bacitracin	(50)	8.6×10^8	2.5 × 10’	2.0×10^9
Bacitracin	(100)	2.1×10^8	1.4×10^7	1.3×10^9
Glutathione	(10)	1.9×10^8	4.8×10^6	8.8×10^8
Glutathione	(50)	2.4×10^8	7.2×10^6	9.7×10^8
Glutathione	(100)	1.0×10^8	8.7×10^5	8.0×10^8
β-NAD	(100)	3.1×10^8	2.3×10^6	9.2×10^8
P-NAD	(500)	8.8×10^8	1.8×10^7	1.7×10^8
β-NAD	(1000)	1.7×10^8	8.5×10^6	1.0×10^9

PKO, wild-type and plasmid pSA1-carrying strain; PK100a, plasmid pSAl.l-carrying strain; PKlOOC, plasmid-free strain; Cys, cysteine; NAD, nicotinamide adenine dinucleotide.
Each value is the average of five cultures.

Table 2. Effect of compounds on the length of aerial mycelium in the plate culture of *Streptomyces azureus* ATCC 14921 (PK0) and its derivatives.

Compounds (μg/ml)		Strain		
		PKO	PKlOOa (μm)	PKlOOC
No addition		69	67	80
L-cys	(10)	72	70	81
L-cys	(50)	100	86	106
L-cys	(100)	98	83	98
D-Cys	(50)	98	74	98
Bacitracin	(10)	124	102	92
Bacitracin	(20)	153	108	107
Bacitracin	(50)	243	96	123
Bacitracin	(100)	170	80	114
Glutathione	(10)	70	68	80
Glutathione	(50)	72	88	88
Glutathione	(100)	67	66	76
β-NAD	(500)	125	80	111
β-NAD	(1000)	111	73	94

Explanations of PKO, PK100a, PK100C, Cys and NAD are the same as described in Table 1.
Each value is the average of about 15 specimens were prepared form five plates.

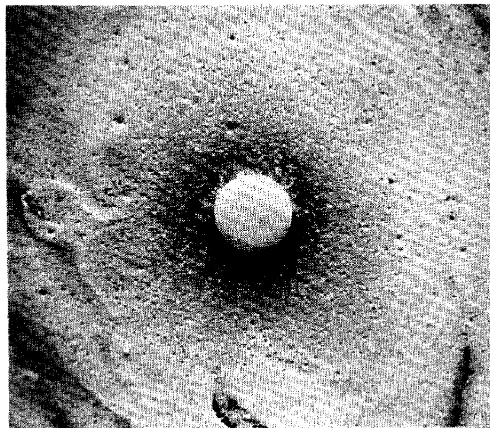

Fig. 1. Stimulation of maturation age of spores.
The coloring of spores or spores mass around the disk contained β-NAD was advanced. Wild-type strain PKO was used in this experiment. Paper disk: 8mm in diameter.

Effect of cysteine, bacitracin, glutathione and P-NAD on the growth of submerged mycelium

Bacitracin and P-NAD showed a growth stimulating effect accompanied by an increase in the submerged mycelium mass, as shown in Table 3. The effect was remarkable and an increase in the amount of mycelium of the strain PKO was 1.3-fold or more than that with no addition at the optimum concentration. The slight or negligible effect was observed for cysteine and glutathione.

Effect of cysteine, bacitracin, glutathione and P-NAD on production of thiostrepton

The effects of cysteine, bacitracin, glutathione and P-NAD on the production of thiostrepton in plate culture were tested. As shown in Table 4, only L- and D-cysteine caused an increase in thiostrepton production for all the strains tested. The effect of L-cysteine was similar to that of D-cysteine. Thiostrepton production of the strain PKlOOa was very low, but it was increased 1.3-fold with L- or D-cysteine at the optimum concentration compared to that with no addition.

Although thiostrepton production of the strain PKlOOa in liquid culture was hardly detectable, it was increased by 10 to 20% of that of strain PKO when the optimal concentration of L- or D-cysteine was 100 μg/ml (Table 5). L- and D-cysteine

Table 3. Effect of compounds on the growth of mycelium in the liquid culture of *Streptomyces azureus* ATCC 14921 (PKO) and its derivatives.

Compounds (μg/ml)		Strain		
		PKO	PKlOOa (mg/ml)	PKlOOC
No addition		52.3	44.6	52.1
L-cys	(10)	53.9	45.7	52.1
L-cys	(50)	59.2	46.0	52.6
L-cys	(100)	53.2	45.2	50.9
D-Cys	(50)	58.5	45.8	52.1
Bacitracin	(10)	58.5	49.2	56.9
Bacitracin	(20)	70.0	50.3	61.7
Bacitracin	(50)	72.0	50.8	58.1
Bacitracin	(100)	51.5	43.1	45.7
Glutathione	(10)	52.4	44.0	52.2
Glutathione	(50)	52.8	44.7	52.5
Glutathione	(100)	52.5	44.3	52.0
P-NAD	(100)	52.3	45.7	61.0
P-NAD	(500)	67.9	51.7	66.7
β-NAD	(1000)	61.6	51.5	67.1

Explanations of PKO, PKlOOa, PK100C, Cys and NAD are the the same as described in Table 1.
Each value is the average of five cultures.

Table 4. Effect of compounds on the production of thiostrepton in the plate culture of *Streptomyces azureus* ATCC 14921 (PKO) and its derivatives.

Compounds (μ g/ml)		Strain (μg/agar plug)		
		PKO	PKlOOa	PKlOOC
No addition		6.4	3.0	4.5
L-cys	(10)	6.5	3.5	4.7
L-Cys	(50)	8.1	4.2	5.3
L-cys	(100)	7.8	3.5	5.3
L-cys	(200)	6.0	3.2	4.3
D-Cys	(50)	7.7	4.2	5.3
D-Cys	(100)	7.5	4.0	5.0
Bacitracin	(10)	6.4	3.0	4.5
Bacitracin	(50)	6.6	3.0	4.7
Bacitracin	(100)	4.0	1.9	2.5
Glutathione	(10)	6.1	3.0	4.5
Glutathione	(50)	6.4	3.0	4.6
Glutathione	(100)	5.1	3.0	3.4
P-NAD	(100)	6.3	3.0	4.5
P-NAD	(500)	6.4	3.0	4.5
P-NAD	(1000)	6.1	3.0	4.1

Explanations of PKO, PKlOOa, PKlOOC, Cys and NAD are the the same as described in Table 1. Plug: 8 mm in diameter and 5 mm in thickness. Each value is the average of five plates.

Table 5. Effect of compounds on the production of thiostrepton in the liquid culture of *Streptomyces azureus* ATCC 14921 (PKO) and its derivatives.

Compounds (μ g/ml)		Strain (μg/w.w.mg of mycelium)		
		PKO	PKlOOa	PKlOOC
No addition		3.0	ND	2.8
L-cys	(10)	3.2	ND	3.0
L-cys	(50)	4.3	0.5	3.5
L-cys	(100)	4.2	0.9	3.6
L-Cys	(200)	2.4	0.3	2.2
D-Cys	(50)	4.0	0.2	3.0
D-Cys	(100)	4.1	0.4	3.3
Bacitracin	(50)	3.0	ND	2.8
Glutathione	(50)	3.0	ND	2.8
β-NAD	(500)	3.0	ND	2.8

Explanations of PKO, PKlOOa, PKlOOC, Cys and NAD are the the same as described in Table 1. ND, not detectable; w.w.mg, wet weight of cells in milligram.
Each value is the average of five cultures.

allowed about 1.4- and 1.3-fold increase in thiostrepton production in strains PKO and PKlOOC, respectively. Bacitracin and β-NAD had no stimulatory effect on thiostrepton production per mycelium. But the production was enhanced in the cultural mass, because the total amount of mycelium was increased in the media (Table 3).

DISCUSSION

Cysteine, bacitracin, glutathione and β-NAD were detected as spore formation-stimulating compounds, which also stimulated the aerial mycelium formation, and the growth of submerged mycelium not only in the pSA1.1-carrying strain PK100a but in the wild-type strain PKO and plasmid-free strain PKlOOC. The first target of each of these four compounds on the cells might differ from one another. However, judging from the known properties or functions of these thiol compounds (Aronson ***et al.,*** 1976; Cheng ***et al.,*** 1973; Garcia-Patrone, 1985) and NAD (Penyige ***et al.,*** 1990), we supposed that they enhanced or changed the cell membrane function, and then stimulated mycelial growth or spore formation in the three strains used. It was also considered that the stimulation of function of the cell membrane overcame the inhibitory effect of pSA1.1, which allowed the strain PK100a to form spores. Similar overcoming effect would be done on the strain PKO, which carried the parent plasmid pSA1, because the stimulatory effect on the strain PKO was greater than that on the plasmid-cured strain PKlOOC. We are eagerly continuing our efforts to clarify the mode of action of bacitracin on the wild-type strain PKO, because this compound showed a distinctly stronger effect on its cell differentiation.

Only cysteine was effective on the stimulation of the thiostrepton production. We understood that it was due to the fact that the structural component of thiostrepton contained one cysteine residue and five thiazole rings derived from cysteine (Anderson ***et al.,*** 1970; Foss, 1988). But bacitracin and glutathione could not be the direct sources of cysteine, although they were cysteine-containing compounds. Racemization was supposed to have occurred from the observation that D-cysteine had a similar effect as that of L-cysteine. Furthermore, the stimulatory effects of mycelial growth and morphological differentiation such as aerial mycelium and spore formation were considered to affect the production of thiostrepton, a secondary metabolite.

We had screened chemicals which overcame the inhibition of spore formation. The chemicals detected were also stimulated both spore formation and mycelial growth (or thiostrepton production) of all strains used. So, the screening method used in this work appears to be an effective method for the screening of substances which stimulate spore formation and mycelial growth or production of antibiotics or secondary metabolites of streptomycetes or which overcome the inhibitory effect of pock-forming plasmids.

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