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Restoration of Aerial Mycelium and Antibiotic Production in a *Streptomyces griseoflavus* Arginine Auxotroph

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An arginine auxotrophic mutant was obtained from *Streptomyces griseoflavus* (bicozamycin-producing strain). The mutant grew on synthetic agar supplemented with either arginine, ornithine, citrulline or argininosuccinate, but produced massive aerial mycelium and bicozamycin only with citrulline. In liquid culture, citrulline also completely restored the ability of the mutant to produce bicozamycin. Culture with arginine or ornithine markedly changed intracellular pools of these ornithine-cycle amino acids, but did not affect the other amino acid pools. The ability to produce antibiotic (but not that to form aerial mycelium) was partially restored by certain mutations to ethionine resistance (Eth-1 and Eth-2). These mutations caused decreased or increased *S*-adenosylmethionine synthetase activity, but both resulted in a 4·5–8-fold increase in the intracellular *S*-adenosylmethionine pool. Exogenous addition of *S*-adenosylmethionine (0·5–3 mM) also partially restored the antibiotic-producing ability of the arginine auxotroph. No difference in the *S*-adenosylmethionine pool was observed in organisms grown with arginine and citrulline. It was suggested that citrulline and *S*-adenosylmethionine are somehow involved in the initiation of differentiation and secondary metabolism of *S. griseoflavus*.

INTRODUCTION

The study of differentiation in *Streptomyces* has been an important topic, especially in industrial microbiology, because this genus produces numerous antibiotics whose production (by secondary metabolism) is known to be linked to differentiation of the producing organisms. When spores of *Streptomyces* are spread on a suitable agar medium, colonies consisting of substrate mycelium appear after incubation for a couple of days, and further incubation results in development of aerial mycelium from the substrate mycelium. The production of antibiotics usually begins at the same time, coupled to differentiation of the mycelium. Using various aerial mycelium-negative (Amy^-) mutants or by biochemical analyses, differentiation of *Streptomyces* species has been studied (Hopwood *et al.*, 1973; Hopwood, 1978; Chater, 1979). It has been suggested that the changes of membrane composition may correspond to the ability of *Streptomyces* mycelium to differentiate (Kalakoutskii & Agre, 1977; Ensign, 1978; Gräfe *et al.*, 1982). The simultaneous disappearance of the arginine auxotrophy (Arg^-) and Amy^- traits has been observed (Redshaw *et al.*, 1979; Nakano *et al.*, 1980; Furumai *et al.*, 1982). Developmental defects resulting from arginine auxotrophy were also reported recently for *Aspergillus nidulans* (Serlupi-Crescenzi *et al.*, 1983). However, little is known about the biochemical events which are responsible for triggering cells to develop aerial mycelium. Recently, the importance of the ornithine cycle in regulation of aerial mycelium formation by *Streptomyces fradiae* was suggested by Vargha *et al.* (1983). In the course of a study of bicozamycin (earlier named bicyclomycin)

Abbreviation: SAM, *S*-adenosylmethionine.

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production by *Streptomyces griseoflavus*, we isolated an arginine auxotroph which can grow with either arginine, ornithine or citrulline, but can produce massive aerial mycelium only with citrulline. As *S*-adenosylmethionine (SAM) has been reported to be involved in the control of sporulation in *Bacillus subtilis* (Ochi & Freese, 1982), we studied the possibility that SAM is also involved in the control of *Streptomyces* differentiation and secondary metabolism. Based on biochemical analyses of mutants we suggest in this paper that citrulline and SAM have an important role in initiation of differentiation and secondary metabolism.

METHODS

Strains and preparation of mutants. *Streptomyces griseoflavus* FERM 1805 (bicozamycin-producing strain) and its derivatives were used (Table 1). The arginine auxotroph, strain AR3, was derived from strain 1805 after treatment of spores with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG). The spontaneous ethionine-resistant mutants (AR3-1, AR3-7 and AR3-10) were obtained as resistant colonies, which developed after 10 d incubation of strain AR3 on synthetic agar (see below) containing 1 mM-arginine and 20 mM-DL-ethionine. (Strains AR3 and 1805 grew slowly with 10 mM-ethionine, but were not able to grow with 20 mM-ethionine.) The ethionine-resistant mutants showed decreased growth rates compared to the parental strain.

Growth conditions. The media used were as follows (compositions are per litre). Synthetic medium: glucose, 20 g; sodium aspartate, 3 g; leucine, 1·3 g; isoleucine, 1·3 g; $MgSO_4$, 0·3 g; NaCl, 1 g; $CaCl_2.2H_2O$, 20 mg; $FeSO_4.7H_2O$, 20 mg; $ZnSO_4.7H_2O$, 20 mg; KH_2PO_4, 1·3 g; $Na_2HPO_4.12H_2O$, 0·36 g; 1 M-MOPS (pH 6·0 by KOH), 70 ml. [Leucine and isoleucine were components of the medium because these amino acids are known to be precursors in bicozamycin biosynthesis (Iseki *et al.*, 1980).] Synthetic agar contained 2% (w/v) agar in addition to the above components. The medium was sterilized by autoclaving at 120 °C for 20 min, apart from the glucose and MOPS solutions, which were sterilized by filtration using Millipore 0·45 μm membrane filters. GPY-medium: glucose, 10 g; polypeptone, 20 g; yeast extract, 5 g; KH_2PO_4, 0·5 g; $MgSO_4$, 0·3 g; NaCl, 0·5 g (adjusted to pH 6·5). GYM agar: glucose, 4 g; yeast extract, 4 g; malt extract, 10 g; Nz-amine (Type A, Wako), 1 g; NaCl, 2 g; agar, 20 g (adjusted to pH 7·0).

Strains were grown in GPY medium (50 ml in 250 ml flasks) at 30 °C for 2 d. Cells were harvested by centrifugation (8000 *g*, 10 min), washed with 100 ml saline, and resuspended in original volume of saline. Washed cells (1 ml) were inoculated into 50 ml synthetic medium (in 250 ml flasks), and cultured at 30 °C on a rotary shaker (230 r.p.m.).

Assay of SAM synthetase. A crude cell-extract was prepared from cells grown in synthetic medium for 24 h, and SAM synthetase activity was measured using [*methyl*-^{14}C]methionine as described by Ochi & Freese (1982), except that incubation was done at 30 °C for 40 min. Protein was measured by the Lowry method, using bovine serum albumin as the standard.

Assay of SAM and amino acid pools. To assay the intracellular SAM pool, organisms were grown in 100 ml synthetic medium plus supplements for 24 h. Cells from 75 ml culture were harvested by filtration (paper diameter 9 cm, no. 2; Toyo Roshi Co.). A separate 25 ml sample was used to determine the dry weight. The filters with cells were quickly inverted onto 7 ml perchloric acid (1·5 M) in a Petri dish. After 1 h incubation at 4 °C, the filter paper was removed by centrifugation (5000 *g*, 5 min), and the remaining cells were removed from the extract by second centrifugation (5000 *g*, 10 min). The supernatant was filtered through a syringe (10 ml) fitted with a Millipore filter (0·45 μm pore size) to remove all remaining debris and then vacuum-evaporated to dryness for 10 h. The residue was dissolved in 1·5 ml deionized water, and insoluble materials were removed by filtration through a syringe (5 ml) fitted with a Millipore filter. SAM assay was carried out using high-performance liquid chromatography (H. Wabiko & E. Freese, personal communication) as follows. The sample (1 ml) was applied to a column of Particil PXS 10/25 SCX (Whatman) which had been washed with 10 mM-KH_2PO_4 (adjusted to pH 4·0). Elution was done at a flow rate of 1·5 ml min^{-1} by a gradient made up of a low ionic strength buffer

Table 1. *Streptomyces griseoflavus strains used*

Strain	Phenotype*	Source
1805	Prototroph, Amy$^+$, Bic$^+$	FERM 1805
AR3	Arg$^-$, Amy$^-$(cond), Bic$^-$(cond)	1805 with NTG
AR3-1	Arg$^-$, Amy$^-$(cond), Bic$^+$, Eth-1	Spontaneous from AR3
AR3-10	Arg$^-$, Amy$^-$(cond), Bic$^+$, Eth-2	Spontaneous from AR3
AR3-7	Arg$^-$, Amy$^-$(cond), Bic$^-$(cond), Eth-3	Spontaneous from AR3

* Amy, ability to form aerial mycelium; Bic, ability to produce bicozamycin; cond, conditional; Eth, ethionine resistance. Each ethionine resistance was designated as Eth-1, Eth-2 and Eth-3 since these resulted in different biochemical properties (see text).

(10 mM-KH_2PO_4, adjusted to pH 4·0 by H_3PO_4) and a high ionic strength buffer (1 M-KH_2PO_4, adjusted to pH 4·0 by H_3PO_4). The percentage of buffer with high ionic strength was increased after 10 min from 0 to 15%, and after a further 15 min from 15 to 40%, and finally remained at 40% for 12 min. UV absorbance at 254 nm was monitored by an UV absorbance detector (Model 440, Waters Associate); SAM eluted after 31 min. The amount of SAM was determined by comparison with the peak area of a standard.

Intracellular amino acid pools were determined as described earlier (Ochi & Freese, 1982).

Assay for bicozamycin. Bicozamycin produced in the liquid or agar medium was determined by a bioassay with *Escherichia coli* strain BS-10 (bicozamycin-sensitive strain, our stock).

Reagents. *S*-Adenosylmethionine (chloride salt, grade II, purity 66%), argininosuccinate (barium salt, purity 90%) and DL-ethionine were purchased from Sigma. All L-amino acids used were of analytical grade.

RESULTS

Isolation and characterization of arginine auxotroph

An arginine auxotroph (AR3) obtained after NTG treatment of wild-type strain (1805) was able to grow on synthetic agar supplemented with either 1 mM-arginine, ornithine or citrulline. This mutant is, therefore, assumed to be deficient in arginine biosynthesis before the ornithine cycle. The mutant grew on GYM agar (nutrient medium) without supplement. Like the parent strain 1805 it formed aerial mycelium and bicozamycin on this medium. In contrast, the mutant produced neither aerial mycelium nor bicozamycin when cultured on synthetic agar supplemented with either 1 mM or lower concentrations of arginine (0·02–0·5 mM). The parent strain (1805) produced both aerial mycelium and bicozamycin, regardless of the presence or absence of arginine (1 mM). Thus, the arginine auxotroph (AR3) showed typically conditional phenotype in aerial mycelium and antibiotic production.

No spontaneous reversion of the mutant strain to Arg^+ was observed (less than 6×10^{-10}), and no Amy^+ strains were detected among more than 10^4 colonies grown on synthetic agar with 1 mM-arginine.

Effect of ornithine cycle compounds on aerial mycelium and antibiotic production

The ability of the Arg^- mutant (AR3) to form aerial mycelium (Amy) and bicozamycin (Bic) on synthetic agar was completely restored by replacing arginine with citrulline, and partially restored with ornithine (Table 2). However, argininosuccinate, a metabolite of citrulline, did not restore bicozamycin production or aerial mycelium formation. [To obtain enough growth, high concentrations of argininosuccinate (i.e. 5 mM) were needed, possibly due to poor uptake.] Glutamate, proline, cysteine, homocysteine or methionine did not restore the ability to produce

Table 2. *Antibiotic, aerial mycelium, and spore formation of strains 1805 and AR3 on synthetic agar*

The spore suspensions from strains 1805 and AR3 were spread on synthetic agar, and incubated at 30 °C for 12 d.

Strains	Supplement (1 mM)	Bicozamycin*	Aerial mycelium†	Spores†
1805 (Parent)	None	+++	+++	+++
	Arginine	+++	+++	+++
AR3 (Arg^-)	Arginine	−	−	−
	Ornithine	+	++	+
	Citrulline	+++	+++	++
	Argininosuccinate‡	−	−	−
	Ornithine + arginine	++	++	+
	Citrulline + arginine	+++	+++	++

* Measured after 7 d culture by placing an agar plug (8 mm diameter, cut from a cultured plate) onto the bioassay plate. −, No antibiotic zone; +, zone 8–14 mm diameter; ++, 14–20 mm; +++, 20–25 mm.

† Measured microscopically after 7 d (for aerial mycelium) or 12 d (for spores). +, Sparse; ++, intermediate; +++, dense.

‡ Added at 5 mM.

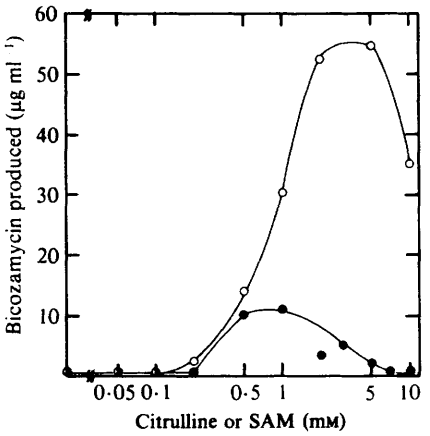

Fig. 1. Restoration of bicozamycin productivity by citrulline or *S*-adenosylmethionine. Strain AR3, grown in GPY medium, was harvested, washed, and then inoculated into synthetic medium supplemented with 5 mM-arginine and various amounts of citrulline (○) or *S*-adenosylmethionine (●). The cultures were grown for 6 d.

aerial mycelium or bicozamycin when added at 1 mM into the medium containing 1 mM-arginine. The restoration by citrulline was not affected by the presence of arginine (Table 2). To study the effect of citrulline in liquid culture, strain AR3, grown in GPY medium, was harvested, inoculated into synthetic medium and cultured for 5 d. When organisms were grown in medium supplemented with arginine (1–5 mM) no bicozamycin was produced either in the medium or intracellularly. However, bicozamycin-producing ability was completely restored by replacing arginine with 5 mM-citrulline or ornithine (bicozamycin production was 60 and 48 μg ml^{-1}, respectively, comparable to 40–56 μg ml^{-1} produced by the parent strain 1805). A lower concentration (1 mM) of citrulline or ornithine was much less effective (2–5 μg bicozamycin ml^{-1}) although this concentration was sufficient for optimal cell growth. Argininosuccinate (5–10 mM) did not restore any bicozamycin production. Restoration of bicozamycin production as a function of citrulline concentration (in the presence of 5 mM-arginine) is shown in Fig. 1. Thus, exogenously added citrulline restored the ability to produce antibiotic in both surface and liquid culture.

Amino acid pools of the parent and the Arg⁻ mutant

Amino acid pools of the Arg^- strain (AR3) cultured in synthetic medium supplemented with arginine, ornithine or citrulline were measured. Cells grown with 5 mM-arginine showed a sevenfold higher arginine pool compared to the parent strain, but the ornithine pool increased only twofold (Table 3). Culture with ornithine drastically increased the ornithine pool (32-fold), whereas culture with citrulline, which completely restored the antibiotic-producing ability, increased the ornithine pool threefold. Unfortunately, the size of the citrulline pool was too small to be estimated, but culture with citrulline should also result in an increase of the intracellular citrulline pool. No marked differences in other amino acid pools were observed between cultures with arginine, ornithine and citrulline.

Effect of mutation to ethionine resistance on aerial mycelium and antibiotic-producing ability

Since it has been suggested that SAM controls differentiation (sporulation) of *Bacillus subtilis* (Ochi & Freese, 1982), we studied whether or not mutations in SAM synthesis could restore the aerial mycelium and antibiotic-producing ability of the arginine auxotroph. For this purpose, we isolated various ethionine-resistant mutants from strain AR3. Among 50 ethionine resistant mutants checked, four strains showed partially restored ability to produce bicozamycin when grown on synthetic agar and in synthetic medium supplemented with 5 mM-arginine, but they

Table 3. *Amino acid pools of strains 1805 and AR3 cultured in synthetic medium*

Strains 1805 and AR3, grown in GPY medium, were harvested, washed, and then inoculated into synthetic medium (supplemented with either arginine, ornithine or citrulline for strain AR3) as described in Methods. After 24 h, cells were harvested and extracted for assay of amino acid pools.

	Amino acid pools [nmol (mg dry wt)$^{-1}$]			
		AR3 grown with 5 mM:		
Amino acid	1805 (parent)	Arginine	Ornithine	Citrulline
Orn	0·15	0·29	4·8	0·43
Cit	ND	ND	ND	ND
Arg	0·35	2·5	0·80	0·30
Gly	0·45	0·82	0·36	0·49
Ala	2·7	2·9	2·1	1·4
Glu	78	69	48	48
Asp	7·0	11	4·5	7·3
Thr	9·0	13	6·4	6·7
Val	71	51	49	45
Leu	11	10	7·4	7·6
Ile	5·2	3·9	3·4	3·0
Lys	2·2	4·1	2·3	2·3

ND, Not detectable.

Table 4. *SAM synthetase and SAM pool of parent and mutant strains cultured in synthetic medium*

Strains were grown in synthetic medium supplemented with arginine, ornithine or citrulline, as in Table 3. SAM synthetase and the SAM pool were assayed as described in Methods.

Strains	Supplement added (5 mM)	SAM synthetase activity* [pmol min^{-1} (mg protein)$^{-1}$]	SAM pool* [pmol (mg dry wt)$^{-1}$]	Bicozamycin produced† (μg ml^{-1})
1805	None	44	ND	40
	Arg	32	73	56
AR3 (Arg$^-$)	Arg	20	24	<1
	Orn	25	25	48
	Cit	22	23	57
AR3-1 (Arg$^-$ Eth-1)	Arg	3·0	190	21
AR3-10 (Arg$^-$ Eth-2)	Arg	61	110	4
AR3-7 (Arg$^-$ Eth-3)	Arg	22	ND	<1

ND, Not determined.
* Assayed using cells grown for 24 h.
† Assayed after 7 d culture.

could not form aerial mycelium. None of 80 strains isolated from the parent (AR3) produced bicozamycin. Thus, in some instances, ethionine resistance (Eth) partially restored the ability of the arginine auxotroph to produce the antibiotic.

We characterized ethionine resistance using three representative ethionine-resistant mutants which did or did not produce bicozamycin (Table 4). An ethionine-resistant mutant (AR3-1, Arg$^-$ Eth-1) showed decreased SAM synthetase activity (one-seventh that of the parent). This mutant regained the ability to produce bicozamycin (21 μg ml^{-1}) in synthetic medium supplemented with arginine. The decreased SAM synthetase activity was not due to intracellular accumulation of an inhibitor, because an equal mixture of the enzyme preparation from the parent and the mutant produced 48% of the SAM synthetase activity obtained with the parent extract alone (data not shown). The SAM pool of the mutant was eightfold higher than

that of parent AR3 (Table 4). A second mutant, AR3-10 (Arg^- Eth-2), regained the ability to produce a small amount of bicozamycin (4 $\mu g\ ml^{-1}$), and produced threefold higher SAM synthetase activity and a 4·5-fold higher SAM pool than the parent. Intracellular methionine pools of the mutants [0·09 and 0·27 nmol (mg dry wt)$^{-1}$ for AR3-1 and AR3-10, respectively] were higher than the parent AR3 [$<0·015$ nmol (mg dry wt)$^{-1}$]. A third mutant, AR3-7 (Arg^- Eth-3), was still negative for bicozamycin-producing ability, and showed SAM synthetase activity as high as that of the parent (AR3). Apparently, ethionine resistance of the mutant resulted from a mutation other than in SAM synthesis (i.e. deficiency in ethionine uptake).

The strain AR3 cultured with citrulline produced essentially the same SAM synthetase activity and had the same SAM pool size as cultures grown in the presence of arginine (Table 4), indicating that citrulline exerted regulation independent of SAM.

Effect of exogenous addition of SAM on antibiotic-producing ability

To study further the possible role of SAM in initiation of antibiotic production, strain AR3 was grown in synthetic medium supplemented with 5 mM-arginine and various amounts of SAM. Exogenous SAM (0·5–3 mM) partially restored bicozamycin production of strain AR3 (Fig. 1). Greater or lower concentrations of SAM were less effective, and growth was partially inhibited by SAM concentrations of 7 mM or higher. Putrescine, spermidine, spermine, methionine or guanosine (added at 0·2, 1 and 5 mM) did not restore the ability to produce bicozamycin, but adenosine partially restored it (5–10 $\mu g\ ml^{-1}$ bicozamycin) when added at a high concentration (5 mM).

DISCUSSION

One of the discriminatory properties of the genus *Streptomyces* is the frequent appearance of arginine auxotrophy and the simultaneous loss of the ability to form aerial mycelium (Sermonti *et al.*, 1978; Redshaw *et al.*, 1979; Nakano *et al.*, 1980). We have shown in this paper that certain arginine auxotrophs from *S. griseoflavus* lose the ability to form aerial mycelium (Amy) and to produce an antibiotic (Bic) on synthetic agar supplemented with arginine. The restoration of antibiotic production and aerial mycelium formation by citrulline should not be attributed to the metabolism of citrulline to arginine or ornithine because culture with citrulline did not markedly increase the intracellular pools of those amino acids (Table 3). Since ornithine can be directly metabolized to citrulline by ornithine transcarbamylase, it is likely that the increase of the ornithine pool also increased the size of the citrulline pool. It is possible that culture of strain AR3 with arginine resulted in a critical decrease in the size of the intracellular citrulline pool due to a genetic deficiency in the ability to synthesize ornithine *de novo*, so that secondary metabolism and differentiation could not be initiated. The possibly important role of citrulline in *Streptomyces* is of interest, as ornithine transcarbamylase activity reaches a maximum during sporulation in *B. subtilis* (Deutscher & Kornberg, 1968).

Although our arginine auxotroph did not show a measurable reversion to Arg^+, it is unlikely that inactivation or loss of an Amy (or Bic) gene occurred in addition to that of the Arg gene, because both the Amy^- and Bic^- phenotypes could be reversed by citrulline.

In addition to citrulline, SAM (or one of its metabolites) seems to be involved in the control of secondary metabolism (possibly initiation) although we can not rule out the possibility that SAM simply contributed to the biosynthesis of bicozamycin. Conceivably, citrulline and SAM exert regulation by different molecular mechanisms.

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