PHYSIOLOGICAL ANALYSIS OF BICOZAMYCIN HIGH-PRODUCING *STREPTOMYCES GRISEOFLAVUS* USED AT INDUSTRIAL LEVEL

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Streptomyces griseoflavus, a bicozamycin-producing wild type strain and its high-producing one derived from it by multiple (>15) mutagenic treatments, were analyzed physiologically and biochemically. The high-producing strain was characterized by: (1) An increased pool size of amino acids including leucine and isoleucine, precursors for bicozamycin synthesis, (2) an earlier and greater accumulation of intracellular ppGpp, (3) a more accentuated decrease in GTP pool size, (4) a higher specific activity of ornithine transcarbamylase which produces citrulline, (5) an increased ability to form aerial mycelium, and (6) an increased resistance to its own antibiotic. We propose that (1), (2) and (4) may be responsible for the high yields of bicozamycin and, possibly, of some other antibiotics produced by *Streptomyces* sp.

One of the distinguishing properties of the genus *Streptomyces* is their abilities to differentiate (*i.e.* aerial mycelium formation and sporulation) and to produce numerous antibiotics.[1,2] Differentiation and secondary metabolism usually occur in parallel in response to nutrient limitation.[3,4] It seems reasonable, therefore, to assume that these two phenomena may partially share regulatory mechanisms, especially with regard to their initiation processes. The pleiotropic loss of multiple functions has been ascribed to a loss of autoregulatory effectors (*e.g.* A-factor,[5~7] factor C[8]) and arginine auxotrophy.[9~11] Although it is recognized that some mutations result in block of cytodifferentiation,[12,13] the biochemical basis for this derangement remains unknown. By isolating and analyzing the relaxed (*rel*) mutants of several *Streptomyces* sp. OCHI has stressed the significance of the stringent response (ppGpp) for their morphological and physiological differentiation.[14~17] The importance of the metabolic control of *Streptomyces* differentiation has been well documented by COLEMAN and ENSIGN,[18] and VARGHA *et al.*[19~22] Also, OKANISHI and KINAMI reported the restoration of kasugamycin production by citrulline in a *Streptomyces kasugaensis* mutant with impaired arginine synthesis.[23]

Bicozamycin (synonym: Bicyclomycin) is a cyclic peptide antibiotic which consists of oxidized leucine and isoleucine[24] and it is commercially important as a feed-additive due to its bactericidal effect on enteric bacteria. Previously, OCHI *et al.* showed that bicozamycin production and aerial mycelium formation by *Streptomyces griseoflavus*, wild type strain 1805, were blocked simultaneously by a mutation to arginine auxotrophy. Both losses could be reversed completely by addition of citrulline.[10] The present report deals with the influence of the stringent response (ppGpp) and ornithine cycle for the initiation of secondary metabolism of *S. griseoflavus* strain 1805 and a bicozamycin high-producing strain derived from it.

Materials and Methods

Strains

S. griseoflavus FERM 1805 is a prototrophic, bicozamycin-producing wild type strain. *S. griseoflavus* 006 is a bicozamycin high-producing strain derived from strain 1805 by sequential mutagenic treatments (>15 times). The latter strain produces bicozamycin more than 300 times as much amount as the former.

Growth Conditions

MG medium, a complex medium for bicozamycin production, contained glucose 10 g, Polypepton 8 g, yeast extract 5 g, KH_2PO_4 0.5 g, $MgSO_4$ (anhydrous) 0.3 g, NaCl 0.5 g and 1 M MOPS 100 ml (adjusted to pH 6.0 with KOH) in 1 liter deionized water. The composition of a 1) synthetic medium, 2) glucose - Polypepton - yeast extract medium (GPY medium), and 3) glucose - yeast extract - malt extract agar (GYM agar) was described previously.[10]

A monoconidial suspension of each strain, prepared by scraping the spores from a GYM agar slant and blending in a vortex mixer with glass beads, was inoculated into GPY medium (50 ml/250-ml Erlenmeyer flask), which was incubated at 30°C on a rotary shaker (250 rpm). After 30 hours incubation, cells (1 ml) were inoculated into MG medium (50 ml/250-ml Erlenmeyer flask), and cultivated at 30°C on a rotary shaker (250 rpm). Cells, grown in GPY medium for 30 hours and previously washed in saline, were inoculated into synthetic medium supplemented with vitamin-free Casamino acids, as described earlier.[10]

Bicozamycin Resistance

Cells of each strain, grown in GPY medium for 30 hours, were inoculated at a 1 : 200 dilution into MG medium containing various amounts of bicozamycin. Growth was observed after 24 hours of incubation with shaking.

Assay of Nucleotide Pool

Nucleotide pools were assayed by means of HPLC after extraction of mycelium with 1 M formic acid, as described previously.[10,25] The intracellular pool size of nucleotides is expressed as pmol or nmol per mg (dry weight) of cells.

Assay of Amino Acid Pool

Cells from 10 to 100 ml culture were quickly harvested by filtration (filter-paper diameter 9 cm, No. 2; Toyo Roshi Co.), extracted with 10 ml of 0.3 M perchloric acid plus 1 mM EDTA as described,[26] and then 50 μl aliquots were applied to an amino acid analyzer (Hitachi Amino Acid Analyzer; Type 835). Amino acids in the pool are expressed as nmol per mg (dry weight) of cells.

Assay of Ornithine Transcarbamylase (EC 2.1.3.3)

A crude enzyme preparation was obtained by sonic disruption (*ca.* 2 minutes) of cells (2 g wet weight) suspended in 5 ml of 10 mM Tris - HCl buffer (pH 7.5). The cellular debris was removed by centrifugation at 15,000×*g* for 20 minutes at 3°C. The supernatant, containing 5 to 15 mg of protein per ml, was used as the enzyme preparation. Ornithine transcarbamylase activity was determined as described by NAKAMURA and JONES.[27] The reaction mixture contained 50 μl of 1 M Tris - HCl buffer (pH 8.5), 25 μl of 0.1 M dilithium carbamyl phosphate, 25 μl of 0.1 M L-ornithine·HCl, 5~20 μl of the enzyme preparation, and deionized water to a final volume of 0.5 ml. The reaction was initiated by addition of ornithine and incubation was run at 37°C for 15 minutes. The reaction was stopped by addition of 1.5 ml of 0.25 N trichloroacetic acid. Citrulline produced in the reaction mixture was measured colorimetrically as described by OGINSKY.[28] Specific activity is expressed as μmol of citrulline formed per hour per mg protein. Protein was assayed by the method of LOWRY *et al.*[29]

Assay of Bicozamycin

The bicozamycin titer was determined by a disc-plate method with *Escherichia coli* strain BS-10 (a bicozamycin-sensitive strain) as a test organism. The assay plate (diameter 8.7 cm) contained 10 ml of Mueller-Hinton medium plus 0.8% agar. The bicozamycin titer was also determined by means of HPLC as follows: A 10-μl sample (supernatant of the cultured broth) was applied directly to a column

(Cosmosil packed column, 4.6×150 mm, Code No. 39047, Nakarai Chemicals Co.) and bicozamycin was eluted at retention time of 8.4 minutes with a solution containing 0.2% tetrahydrofuran and 15 mM phosphoric acid at a flow rate of 1.5 ml/minute and absorbance was measured at 210 nm.

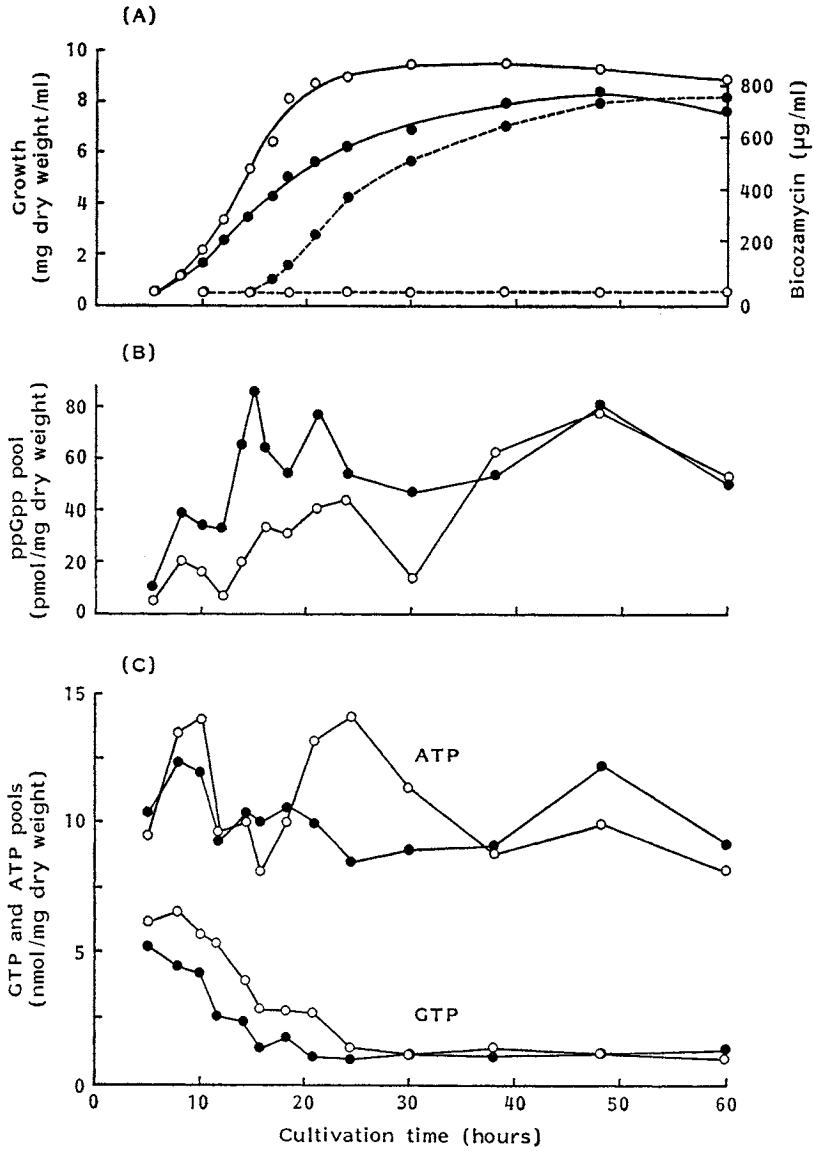
Results

Bicozamycin Production by the Wild Type and High-producing Strains

Bicozamycin synthesis (60 μg/ml) by the wild type strain in a chemically defined medium was

Fig. 1. The time course of bicozamycin production and changes in ppGpp, GTP, and ATP pools of *Streptomyces griseoflavus* strains cultivated in MG medium.

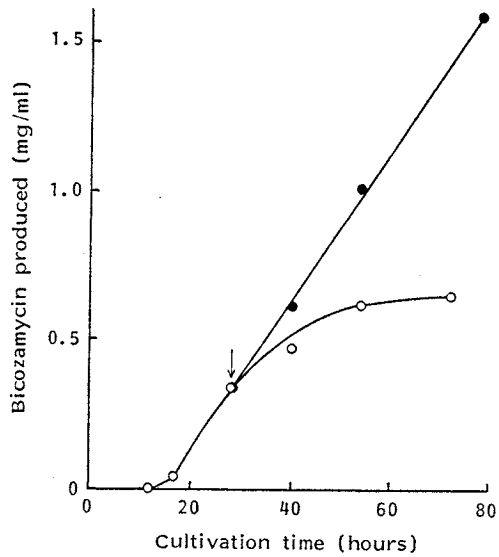
○ Wild type strain 1805, ● bicozamycin high-producing strain 006, — growth, ---- bicozamycin.



All the experimental procedures were described in Materials and Methods. The data were all from the same experiment.

inhibited completely (<0.5 μg/ml) by addition of 2% (weight/volume) Casamino acids, in similar to the other antibiotic fermentations by *Streptomyces* sp.[14~16] In addition, the wild type strain produced only 1~2 μg/ml of bicozamycin in MG medium, a nutritionally rich medium containing 0.8% Polypepton. In contrast, the high-producing strain produced a large amount of bicozamycin (800 μg/ml) in MG medium. Its formation was initiated at 14 hours after inoculation and rapid synthesis of bicozamycin was observed within a short period of time (10 hours) (Fig. 1A). Thereafter the rate of antibiotic synthesis declined significantly. This may be due to a limitation of precursors *per se* required for bicozamycin synthesis; addition of both leucine and isoleucine, known precursors for bicozamycin synthesis,[24] at 28 hours, markedly stimulated antibiotic production, without affecting the growth rate or yield of biomass (Fig. 2). Thus, the availability of a larger supply of precursors (including indirect precursors) appeared to be an essential factor for high productivity of the antibiotic. We, therefore, assumed that the high-producing strain may harbor genetic factor(s) responsible for a greater pool size of amino acids, especially leucine and isoleucine. This was confirmed by using cells harvested at various growth phase. As shown in Table 1, the high-producing strain maintained the total amino acid pool size 20~70% higher than those of the wild type strain throughout the cultivation. The larger pool for amino acids not just leucine and isoleucine in the former than the latter may contribute to the greater synthesis of bicozamycin as a source of indirect precursors. The pool sizes of leucine and isoleucine in the former were 3- to 6-fold higher than the later after 8 hours cultivation. Later (*e.g.* 24 hours), the pool sizes of leucine and isoleucine (and some other amino acids) of the former were even lower than the latter. This may be explained by a rapid utilization of those amino acids for bicozamycin synthesis. Since the wild type strain still failed to produce bicozamycin even with leucine and isoleucine supplementation it is suggested that the high-producing strain may also harbor mechanism(s) by which 'initiation processes' of the antibiotic synthesis are accelerated.

Fig. 2. Bicozamycin production by strain 006.
● Plus leucine and isoleucine, ○ control.



Cells of strain 006 were grown in the same medium as in Fig. 1. After 28 hours of incubation the culture was divided into two flasks and leucine and isoleucine were added to one of them each at final concentration of 0.2%, and then incubation was further continued for 72 hours. The arrow indicates the time of addition of leucine and isoleucine.

Nucleotide Pool Changes during Cultivation

It has been postulated that ppGpp may be a signal molecule which triggers (or is needed for) antibiotic synthesis.[14~16] We, therefore, measured the changes in ppGpp pool size during cultivation. As shown in Fig. 1B, the pool size of ppGpp (strain 1805) increased gradually and was maximal at 24 hours when cells had just entered the stationary phase. Interestingly, the ppGpp pool size of the high-producing strain 006 was about 2- to 3-fold greater than that of the wild type strain 1805. It should

Table 1. Amino acid pools of strains 1805 and 006 grown in MG medium.
Cells cultivated for indicated times were harvested and extracted for assay of amino acid pools. The results are from the same experiment in Fig. 1.

Amino acid	Amino acid pools (nmol/mg dry weight)					
	8 hours		14 hours		24 hours	
	1805	006	1805	006	1805	006
Aba[a]	7.05	14.3	3.79	6.97	0.64	0.38
Ala	9.48	5.35	3.31	2.38	1.89	3.70
Arg	0.93	2.39	0.37	0.57	0.20	0.29
Asp	1.73	ND	4.18	3.13	2.71	3.16
Cys	0.42	0.48	0.33	0.28	0.52	1.11
Dap[b]	0.85	1.13	0.37	0.60	1.47	13.2
Gln	31.5	76.1	51.5	39.1	23.7	35.4
Glu	117	142	136	177	83.0	151
Gly	0.25	5.50	0.73	3.10	ND	0.13
His	0.41	1.17	0.10	0.19	0.09	ND
Ile	0.67	4.01	0.47	0.63	0.51	0.14
Leu	4.92	15.3	1.58	3.94	0.70	0.52
Lys	1.21	4.06	4.26	9.66	1.95	0.54
Met	3.10	4.78	2.75	3.46	0.51	0.28
Orn	0.21	0.79	0.41	0.80	0.66	0.40
Phe	1.52	3.38	2.46	3.22	0.64	0.21
Ser	0.94	2.82	1.41	1.43	3.18	1.09
Val	2.25	17.3	7.10	5.69	1.03	0.81
Total	184	301	221	262	123	212

[a] γ-Amino butyric acid. [b] α,ε-Diaminopimelic acid.
ND: Not detected.

be pointed out that the onset of bicozamycin production (Fig. 1A) appears to coincide to the maximal accumulation of ppGpp. A decrease in the GTP pool size (Fig. 1C) occurred concomitantly with increase in ppGpp. No marked difference between the wild type and high-producing strains was observed in the pool size of ATP (Fig. 1C), UTP or CTP (not shown). The accumulation of ppGpp at higher levels in the mutant was also observed by the experiment using MG medium containing as much as 2% Polypepton. Thus, it is suggested that the high-producing strain, as a consequence of one or more mutational events, has the ability to accumulate higher levels of ppGpp.

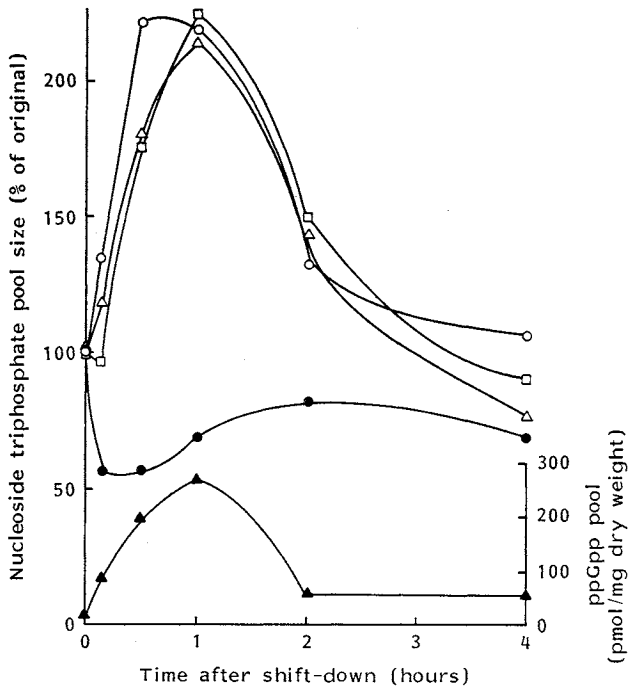
We also investigated how *S. griseoflavus* accumulated ppGpp. When the wild type strain, grown to the mid-exponential growth phase in the chemically defined medium supplemented with 2% Casamino acids, was transferred to the fresh chemically defined medium without Casamino acids, there was an immediate accumulation of ppGpp after shift-down (Fig. 3). Apparently, *S. griseoflavus*, like other *Streptomyces* sp.,[14~16] accumulates ppGpp in response to amino acid starvation. Later, *i.e.*, 2~4 hours after transfer, ppGpp pool size decreased (though still higher than the initial level), possibly due to adaptation of cells to *de novo* synthesis of amino acids. Similar experiments was not applicable to the high-producing strain because it grew too slowly in the Casamino acid medium mentioned above.

Ornithine Transcarbamylase Activity during Cultivation

It has been postulated that citrulline positively controls the initiation of bicozamycin production.[10] We investigated further the putative role of this amino acid in the high-producing strain.

Fig. 3. Changes in the intracellular pools of nucleoside triphosphates and ppGpp after nutritional shift-down.

▲ ppGpp, ● GTP, ○ ATP, □ UTP, △ CTP.



Cells of strain 1805 were grown to mid-exponential phase, 10 hours after inoculation, in the chemically defined medium supplemented with 2% (weight/volume) vitamin-free Casamino acids and transferred to the fresh chemically defined medium without Casamino acids. Nucleotide pools were determined as described in Materials and Methods. Just before transfer, nucleotide pools of GTP, ATP, UTP and CTP were 3,430, 9,250, 3,950 and 2,770 pmol/mg dry weight, respectively.

In similar to previous report for the wild type strain,[10] the citrulline pool size was also undetectable for strain 006. We, therefore, analyzed the dynamic aspect of the synthesis of ornithine transcarbamylase (OTCase) which produces citrulline from ornithine and carbamyl phosphate. In both strains, specific activity of OTCase was quite low in young cultures, but its synthesis was abruptly derepressed at 10 hours (Fig. 4) as similarly reported for *Bacillus subtilis*.[30] Derepression of OTCase formation, particularly striking in the high-producing strain, was about 4-fold greater than that observed in the wild type strain (Fig. 4). Interestingly, the derepression of enzyme synthesis took place just prior to initiation to bicozamycin production. Since pool size of the substrate (ornithine) of the high-producing strain at 14 hours was greater than that of the wild type strain (Table 1), the former strain could produce more than 4-fold amount of citrulline at this time.

Characterization of Ornithine Transcarbamylase

In the wild type strain, OTCase was repressed completely to the basal level when cells were grown in MG medium supplemented with 1 mM arginine (Fig. 5). In contrast, the high-producing strain produced OTCase at high levels in the medium containing up to 2 mM arginine, but its production was fully repressed to basal levels by addition of 5 mM arginine. Thus, OTCase synthesis in the high-producing strain is partially, but not completely, desensitized to end-product repression. Although

Fig. 4. Changes in specific activity of ornithine transcarbamylase (OTCase) during growth in MG medium.

○ Strain 1805, ● strain 006.

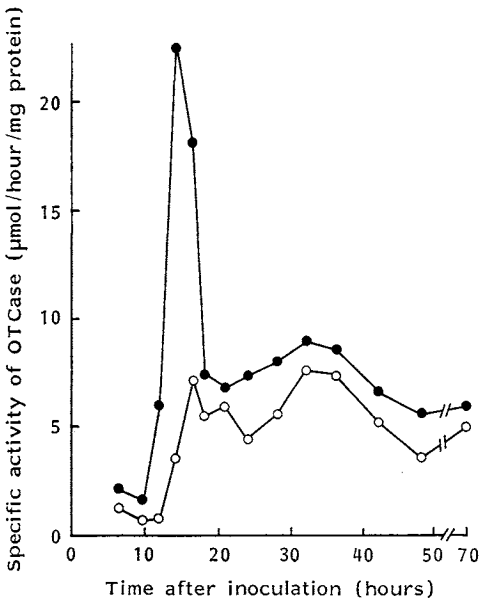
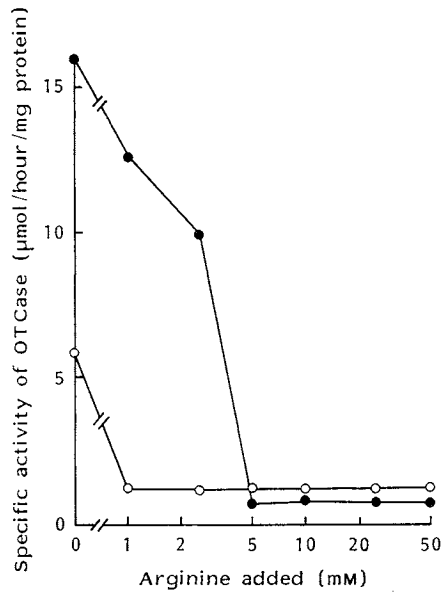


Fig. 5. Repression of ornithine transcarbamylase (OTCase) by arginine.

○ Strain 1805, ● strain 006.



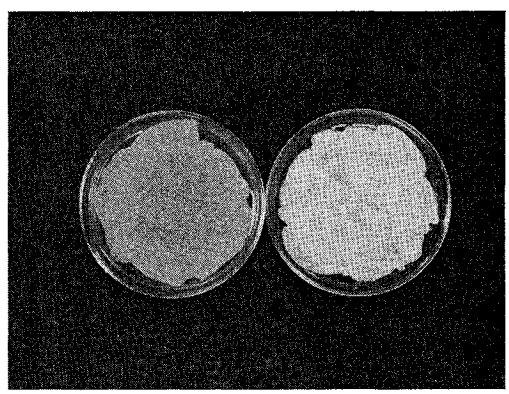
Cells of strains 1805 and 006 were grown in MG medium supplemented with various amounts of arginine, and OTCase activity was determined after 16 hours of cultivation.

OTCase of the wild type strain was also subject to inhibition by arginine, its inhibition was weak, *i.e.* 38% inhibition by 10 mM arginine in the presence of 1 mM ornithine.

Ability to form Aerial Mycelium

Both the wild type and high-producing strains developed aerial mycelium after 3 days of inoculation on GYM agar. Since the GTP pool size decreased more rapidly in the high-producing than in the wild type strain as examined in the liquid culture (Fig. 1C), it was reasoned that the high-producing strain might exhibit a greater ability to form aerial mycelium.[31] As expected, development of aerial mycelium by the wild type strain was completely inhibited during 10 days' incubation when cells were grown on GYM agar supplemented with 4% Casamino acids; in contrast, the high-producing strain still produced aerial mycelium even with 5% Casamino acids in the medium. Complete inhibition was only observed with 7% Casamino acids. However, Casamino acid-suppressed culture of the wild type strain was able to induce formation of aerial mycelium and also

Fig. 6. Reversal of aerial mycelium formation by addition of decoyinine in *Streptomyces griseoflavus* strain 1805 on a plate containing excess amount of Casamino acids.



Spores were spread on GYM agar (plus 100 mM MOPS, pH 7.0 with KOH) supplemented with 4% vitamin-free Casamino acids without (left) or with (right) 0.3 mM decoyinine. The plates were incubated at 30°C for 7 days.

eventually to sporulate by addition of 0.1~0.3 mM decoyinine, a specific inhibitor of GMP synthetase (Fig. 6), in similar to that seen with other *Streptomyces* sp.[16,31]

Resistance to Bicozamycin

Resistance of *S. griseoflavus* to bicozamycin (self-resistance) was also examined. The high-producing strain showed 5-fold greater resistance than the wild type strain (10 mg vs. 2 mg/ml of bicozamycin to attain a 50%-growth inhibition, respectively).

Discussion

On the contrary to the impaired ability of micro-organisms to produce antibiotics caused by *rel* mutation or mutation to arginine auxotrophy, it was shown in this study that a dramatically increased ability to produce bicozamycin was associated with increase in specific activity of ornithine transcarbamylase and accumulation of ppGpp. The high-producing strain is the mutant strain carefully selected to the level of industrial use. Therefore, we suggest that the observed physiological properties of the high-producing strain are a consequence of the mutations and strain selections and might be responsible for the enhanced production of bicozamycin. Although this hypothesis is an attractive one, the results described herein do not provide an absolute correlation between them. The mechanism of initiation of antibiotic production may involve certain regularoty systems or processes. Thus, regulatory mutations could result in an acceleration of transcriptional events leading to initiation and expression of biosynthetic genes of the antibiotic. From these points of view, we have previously shown that the introduction of relaxed (*rel*) mutations into *Bacillus subtilis* (2 strains) and *Streptomyces* (3 species) results in loss of the ability to produce antibiotics.[14~16,32] As will be reported elsewhere, a *rel* mutant derived from *S. griseoflavus* wild type strain, obtained as a spontaneous thiopeptin-resistant isolates, also lost the ability to produce bicozamycin. From the study of stepwise enhancement of α-amylase production, MARUO *et al.* emphasized the importance of synergistic rather than additive effects of the pre-existing and added genetic factors.[33,34] In the light of these findings one should recognize with respect to strain improvement that each genetic factor can exert its maximal effect only through interactions with other genetic factors.

An increase in ability to accumulate intracellular ppGpp could arise from either its increased synthesis or decreased degradation, or a combination of these two processes. A ppGpp is synthesized in response to amino acid starvation by the action of *relA* gene product (stringent factor) and uncharged tRNA, using ATP and GTP as substrates.[35] Increased synthesis of ppGpp, therefore, could arise from mutations which depress transport of amino acids or its *de novo* synthesis, or from those which interfere with amino acid-charging of tRNA, or from those which alter ribosomal machinery *per se*. On the other hand, decreased degradation could result from mutations which depress activity of protein(s) involved in degradation. Such a mutation, designated *spoT*, is known in *E. coli*.[35] Since turnover of ppGpp is very rapid, 20 seconds in *E. coli*,[36] the impeded degradation may be very effective for increase in ppGpp accumulation. Indeed, a *spoT* mutant was shown to maintain 2- to 3-fold higher concentration of ppGpp than a *spoT*$^+$ strain.[36] In addition to the well-known mechanism of ppGpp synthesis described above, ribosome-independent or stringent factor-independent ppGpp synthesis is also known.[37~39] It remains unknown whether such a novel type of ppGpp synthesis contributed or not to the observed increase in the ppGpp pool size during cultivation of the high-producing strain. The mechanism by which the ppGpp pool size of the wild type strain increased during late stationary phase (Fig. 1B) also remains obscure. However, contribution of the increased ppGpp pool for initiation of the antibiotic production may be negated in this strain by the limited nutrient and precursor pools.

The most important thing for obtaining readily a desired mutant is the availability of a selection procedure(s) or facile detection method. In this regard, the increased ability of the high-producing strain to form aerial mycelium may offer an intriguing possibility as a screening procedure. Conceivably, aerial mycelium formation may partially be due to a severe inhibition of GMP (and GTP)

synthesis by ppGpp, which leads to the onset of differentiation. This hypothesis is consistent with the facts that Casamino acid-repression of aerial mycelium formation was completely reversed by addition of decoyinine (Fig. 6) and that a *S. griseoflavus rel* mutant showed a reduced and dramatically delayed appearance of aerial mycelium (unpublished). Therefore, mutants with increased ability to accumulate ppGpp may be detected at high frequency by selection of colonies which develop aerial mycelium on nutritionally rich media.

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