BIOSYNTHESIS OF BICYCLOMYCIN
I. APPEARANCE OF AERIAL MYCELIA NEGATIVE STRAINS (am$^-$)

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The degeneration of bicyclomycin-producing strains of *Streptomyces sapporonensis* resulted in sharply depressed bicyclomycin formation in a large scale fermentation. Degenerated strains, whose productivities were only 1/30 to 1/100 of normal strains, could not form aerial mycelia on glucose-BENNETT's agar; they were aerial mycelia negative strains (am$^-$). Repeated transfers of culture, treatment of mycelia with acriflavin, mechanical agitation shock on mycelia or higher growth conditions stimulated the degeneration of producing strains, suggesting the involvement of extrachromosomal elements or plasmids in biosynthesis of bicyclomycin.

Shake flask fermentations inoculated with a mixture of a normal high-producing strain and a degenerated low-producing strain resulted in sharply depressed bicyclomycin formation in proportion to the increase of low-producing strain added. It appears that the low-producing strain outgrew the high-producing strain.

Bicyclomycin was discovered from the culture broth of *Streptomyces sapporonensis*[1] and several other strains of *Streptomyces*.[2]

During the investigation of production of bicyclomycin by *Streptomyces sapporonensis*, degeneration of the producing strain, especially in the course of a large scale fermentation, was observed. The producing strain degenerated to a low-producing strain, which had lost the ability to form aerial mycelium (am$^-$ strain).

This paper describes the characteristics of am$^-$ strains and the stimulatory conditions leading to the formation of the strains.

Materials and Methods

Organisms and media

Strain LH9455 was an improved mutant obtained from the wild-type strain of *Streptomyces sapporonensis*. Strain 30, 47 and 49 were isolated from 4 m^3 tank fermentation broth of strain LH9455. Strain 38 (am$^+$, pro$^-$), proline-requiring mutant, was derived from strain LH9455 by UV-irradiation. Strain 38 (am$^-$, pro$^-$) and 38 (am$^-$, pro$^+$) were derived from strain 38 (am$^+$, pro$^-$) and 38 (am$^+$, pro$^+$), respectively, by incubation in the presence of glass beads. The organisms were maintained on BENNETT's agar slants. Production medium contained (per liter) 20 g potato starch, 20 g cotton seed meal (Pharmamedia), 10 g gluten meal, 5 g $MgSO_4 \cdot 7H_2O$, 10.9 g KH_2PO_4 and 7.2 g $Na_2HPO_4 \cdot 12H_2O$.

Culture conditions

Seed cultures inoculated with spores from a slant culture were grown at 30°C for 2~3 days in 100 ml Erlenmeyer flasks containing 20 ml of production medium on a rotary shaker with a 3-inch throw, at 220 rev./min. Fermentations were conducted in 100 ml Erlenmeyer flasks containing 20 ml of production medium. The flasks were inoculated with 5% (v/v) of a vegetative inoculum and incubated at 30°C for 2~7 days on a rotary shaker with a 3-inch throw and 220 rev./min.

To test the effect of repeated transfers of inoculum, the seed flasks were incubated at 30°C for

3 days when 5% of the culture was transferred into new flasks containing the production medium for a new incubation under the same conditions. The transfer procedure was repeated every third day taking the inoculum from the previous incubation. For model experiments of mechanical agitation shock, the flasks contained 10 glass beads (20 mg, 3 mm diameter), 2 baffle plates (30×10×1 mm) or 2 glass rods (20 mm, 5 mm diameter). For experiments with curing agents, transfer of culture was performed in the presence of glass beads and curing agent. For experiments with acriflavin, flasks containing production medium with 10 glass beads in the presence (AF (B)) or absence (B) of acriflavin (50 μg/ml) were inoculated with the seed broth with (AF (B)) or without (B) acriflavin (50 μg/ml).

Isolation of aerial mycelia negative (am^-) colonies

An appropriate quantity of culture broth was homogenized aseptically with a Waring blender for 10 minutes. Aliquots (0.1 ml) of serial dilutions in 0.8% NaCl solution of the homogenate were spread on the surface of glucose-BENNETT's agar plates, which were incubated at 30°C for 5~7 days. Am^- colonies were counted among at least 200 colonies.

Antibiotic assay

Mycelia were removed from fermentation broth by filtration. The antibiotic titer of the resulting filtrate was determined by the standard disc-plate technique with *E. coli* ATCC 27166 as an assay organism. For determination of the bicyclomycin productivity of an am^- colony, the colony grown on BENNETT's agar at 30°C for 5~7 days was cut out with a cork borer and the agar piece was plated on a bioassay plate containing *E. coli* ATCC 27166.

Results

1. Appearance of Aerial Mycelia Negative Strains

As shown in Fig. 1, the depression of bicyclomycin production was observed in 4 m^3 scale fermentation (133 rpm, two stages of disc turbine impellers with six blades). To elucidate the cause, culture broth was homogenized, diluted serially and plated on glucose-BENNETT's agar on each day of the fermentation to check the bicyclomycin productivity of the organism. About half of the colonies obtained

Fig. 1. Time course of bicyclomycin production and appearance of aerial mycelia negative (am^-) colonies.

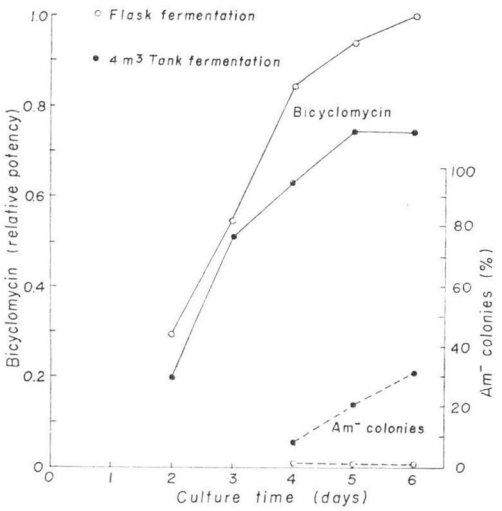


Fig. 2. Bicyclomycin productivity of strains isolated from tank culture broth.

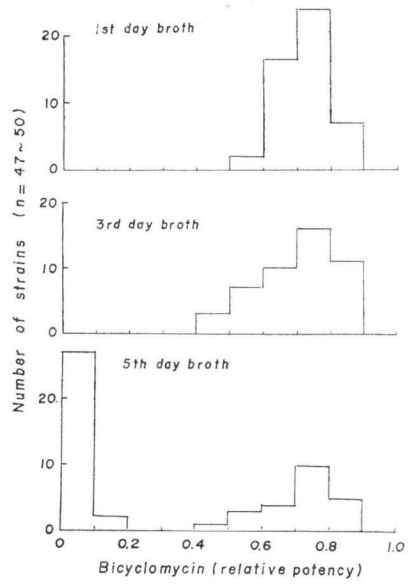


Fig. 3. Effect of glass beads, baffle plates or glass rods on the formation of am⁻ colonies.

Strain LH9455 was cultured at 30°C in flasks containing 10 glass beads, 2 baffle plates or 2 glass rods.

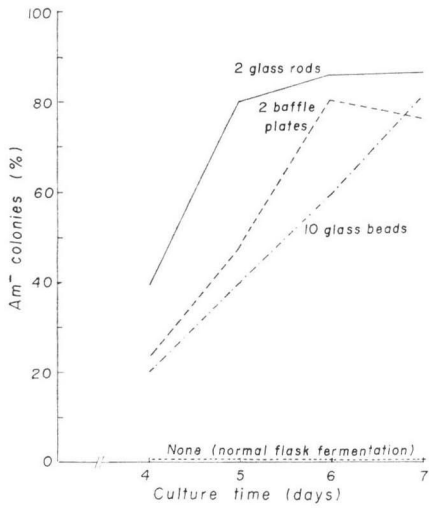


Fig. 4. Effect of repeated transfer of inoculum on appearance of am⁻ colonies.

After transfer of inoculum one (S1), two (S2), three (S3) or four (S4) times, bicyclomycin fermentation was conducted in flasks containing production medium as described in Materials and Methods.

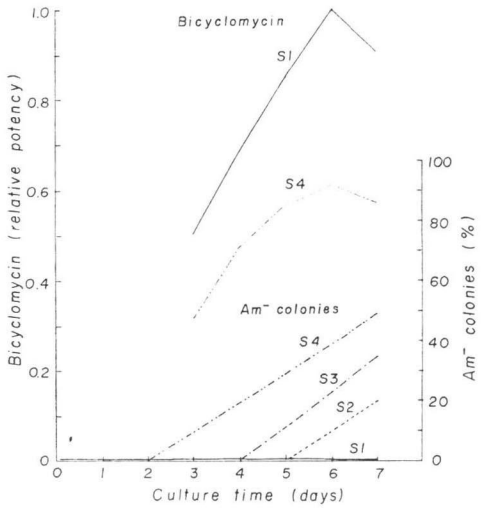
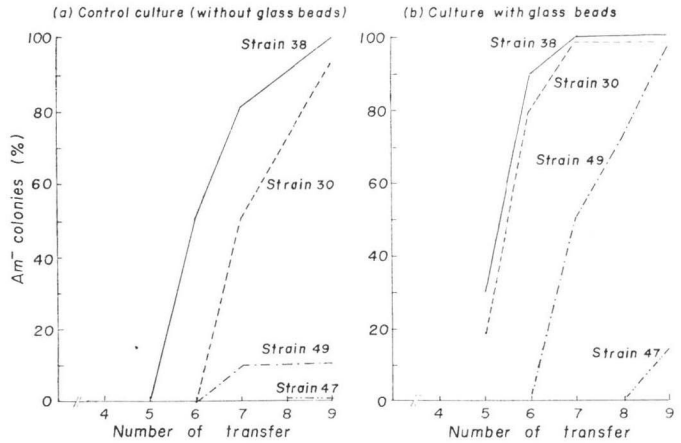


Fig. 5. Effect of glass beads and repeated transfer on appearance of am⁻ colonies.

The strains 30, 49 and 47 were isolated from 4 m³ tank fermentation broth. The transfer of culture in the medium for production was performed in the presence or absence of glass beads and the number of am⁻ colonies were counted at the time of each transfer.



from the fifth day broth, at which time bicyclomycin production in the fermentation had reached a plateau, had low productivity, and almost all of them could not form any aerial mycelium; they were aerial mycelia negative (am⁻) strains on glucose-BENNETT's agar (Fig. 2). However, the depression of bicyclomycin production did not occur in a flask fermentation on a rotary shaker. It was thus surmised that the depression of production in a large scale or agitated fermentation was due to the appearance of am⁻ strains.

2. Conditions for Appearance of am$^-$ Strains

To elucidate the cause of appearance of am$^-$ strains, the conditions for their formation was examined in flask fermentations with glass beads, baffle plates or glass rods. No am$^-$ colonies were formed in the normal culture (Fig. 3), while they did appear in the fourth day cultures containing 10 pieces of glass beads, 2 baffle plates or 2 glass rods. The frequency of appearance of am$^-$ colonies reached 80~90% on the seventh day. Thus, it was evident that mechanical shock on mycelia stimulated the appearance of am$^-$ colonies.

Table 1. Effect of curing agents in repeated transfers of inoculum on appearance of am$^-$ strains.

Transfer of culture was performed in the presence of glass beads and curing agent. Formation of am$^-$ colonies was determined after four transfers.

Curing agent μg/ml	am$^-$ colonies (%)
None (control)	8.2
Ethidium bromide, 50	19.2
Acriflavin, 50	88.5
Sodium dodecyl sulfate, 50	19.2

The effect of repeated transfer of inoculum on appearance of am$^-$ colonies was examined. As shown in Fig. 4, am$^-$ colonies were formed and bicyclomycin production was depressed in proportion to the number of transfers of inoculum. In the fourth transfer of inoculum, am$^-$ colonies appeared in the second day culture, and bicyclomycin production was depressed by 40% compared with the first transfer.

Fig. 5 shows the appearance of am$^-$ colonies with the strains isolated from 4 m^3 tank fermentation broth. As transfers were repeated, it became apparent that the formation of am$^-$ colonies was stimulated in strains 38 and 30, which had higher growth rates than the other two strains. When the cultures were transferred in the presence of glass beads, the frequency of appearance of am$^-$ colonies reached 80~90% at the sixth transfer. Also in the case of strain 49, in spite of its low growth rate, the appearance of am$^-$ colonies was markedly stimulated on repeated transfers of inoculum, whereas strain

Fig. 6. Effect of acriflavin and glass beads on appearance of am$^-$ colonies.

Flasks containing production medium with 10 glass beads in the presence (AF (B)) or absence (B) of acriflavin (50 μg/ml) were inoculated with the seed broth with (AF(B)) or without (B) acriflavin (50 μg/ml) and were incubated at 30°C.

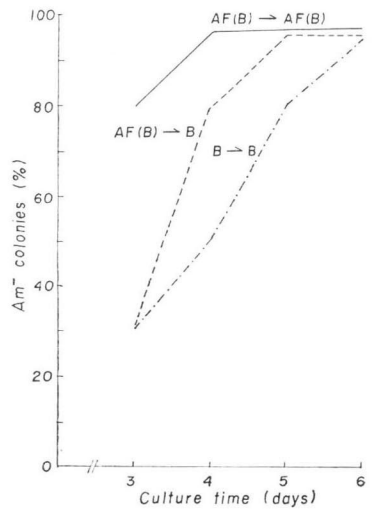


Fig. 7. Effect of growth temperature on appearance of am$^-$ colonies.

After the seed flasks had been incubated for 3 days at 30°C or 40°C, production medium was inoculated and incubated at 30°C or 40°C.

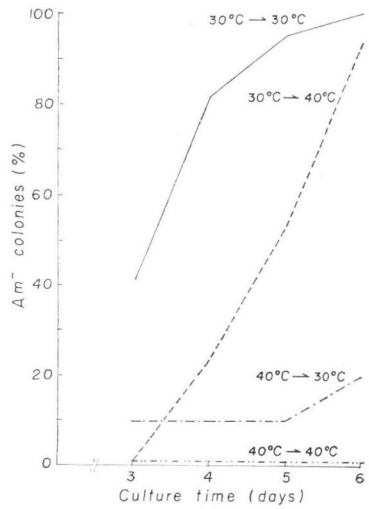


Table 2. Culture characteristics of am⁻ strains.

The strains were classified according to their ability to form aerial mycelium on glucose-, starch-, or galactose-BENNETT's agar. Bicyclomycin productivity of am⁻ strain was expressed as relative potency compared to that of the normal strain.

Type	Aerial mycelium formation on BENNETT's agar			Bicyclomycin productivity relative potency (%)	Isolation
	Glucose	Starch	Galactose		
Normal	+	+	+	100	
A	±	+	+	3.0	without beads
B	−	+	±	1.0	without beads, or beads, or acriflavin
C	−	−	−	3.0	beads, acriflavin
D	−	+	±	3.0	

Fig. 8. Effect of starch and glucose as carbon source in repeated transfers of inoculum on appearance of am⁻ strains.

The transfers of inoculum were done in the presence or absence of 10 glass beads, or in the presence of acriflavin (50 μg/ml). The number of am⁻ colonies were counted at the time of each transfer.

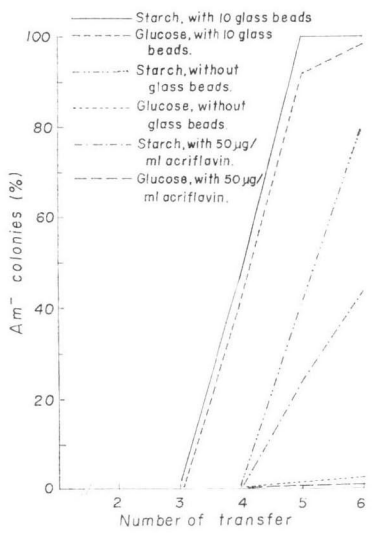
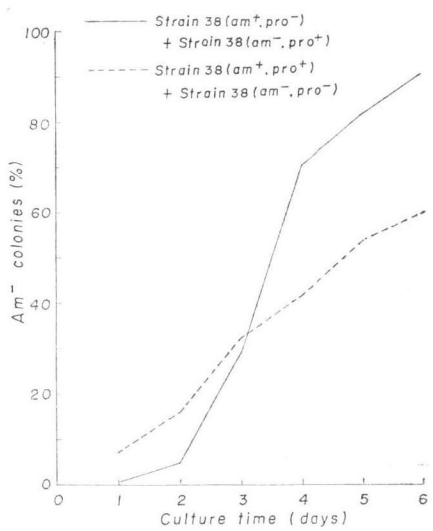


Fig. 9. Frequency of am⁻ colonies in mixed culture of a normal strain and an am⁻ strain.

Shake flask fermentation was inoculated with an equal volume of a culture of a normal strain and an am⁻, low-producing strain.



47 was affected only slightly.

The effect of acriflavin on the formation of am⁻ colonies was examined. As shown in Fig. 6, the frequency of appearance of am⁻ colonies was the highest in the condition, in which both seed and main culture contained both acriflavin (AF) and glass beads; the frequency was 80% on the third day. Without glass beads in the culture, the frequency was low (Data not shown). The effect of agents known to cause curing of plasmids was then examined in the case of four repeated transfers of culture in the presence of glass beads. Acriflavin gave 88.5% am⁻ colonies. Other curing agents, ethidium

Fig. 10. Mixed culture of normal and am⁻ (type B) strains.

Shake flask fermentation was inoculated with a normal strain and an am⁻, low-producing strain in different proportions. The formation of am⁻ colonies and bicyclomycin production was examined in the mixed culture.

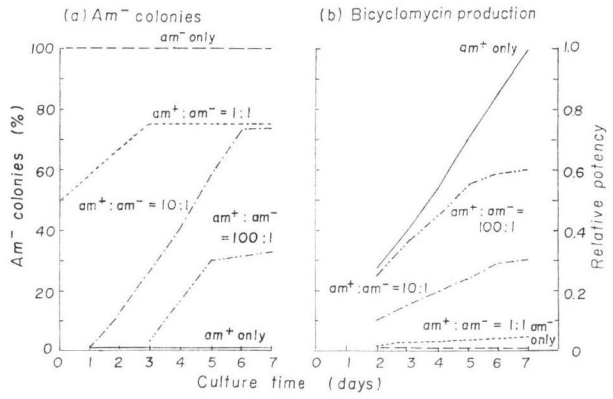
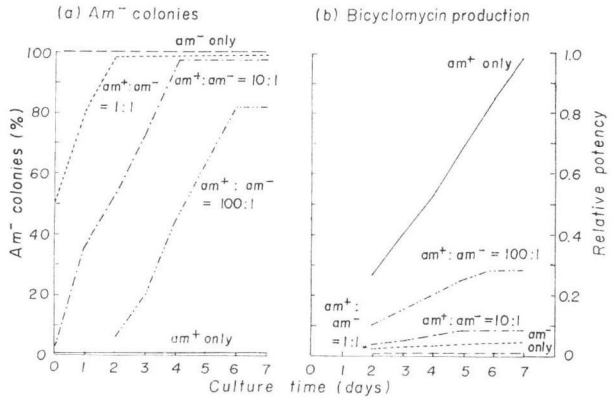


Fig. 11. Mixed culture of normal and am⁻ (type C) strains.



bromide and sodium dodecyl sulfate, had, however, only a weak effect even if the culture contained glass beads (Table 1).

The appearance of am⁻ colonies was also stimulated by better growth conditions. In case of transfer at 30°C, the optimum growth temperature, the appearance of am⁻ colonies was stimulated, whereas it was not at 40°C, the critical temperature for growth (Fig. 7). *St. sapporonensis* grew better with starch than with glucose as a carbon source. In the presence of glass beads, the appearance of am⁻ colonies was the same in both production media. However, in the culture without glass beads, am⁻ colonies were formed only in the starch-medium but not in the glucose-medium (Fig. 8).

3. Characteristics of Aerial Mycelia Negative (am⁻) Strains

Characteristics of am⁻ strains different from those of the normal parent strain were as follows: they formed small colonies on glucose-BENNETT's agar, especially their growth was delayed, they were scarcely capable or totally incapable of forming aerial mycelia, and their bicyclomycin productivity was 1/30 to 1/100 of the normal strain. These characteristics were genetically stable. As shown in

Table 2, the strains could be classified into four different types according to their ability to form aerial mycelium and their morphology on BENNETT's agar with different carbon sources. The am$^-$ strains belonging to type A, B and D were able to form aerial mycelium on starch-BENNETT's agar, whereas those of type C could not form aerial mycelium on any BENNETT's agar. Am$^-$ strain of type B appeared very frequently under every condition described above.

4. Mixed Culture of Normal Strain and am$^-$ Strain

When shake flask fermentations were inoculated with a normal strain 38 (am$^+$, pro$^-$) together with an am$^-$, low-producing, strain 38 (am$^-$, pro$^+$), or a normal strain 38 (am$^+$, pro$^+$) together with an am$^-$, low-producing, strain 38 (am$^-$, pro$^-$), the am$^-$ colonies formed were of the same phenotype as that of the am$^-$ strain added (Fig. 9). These results indicated that transformation from am$^+$ to am$^-$ strain or from am$^-$ to am$^+$ did not occur, and that the am$^-$ strain outgrew the normal am$^+$ strain.

Fig. 10 shows the correlation between appearance of am$^-$ colonies and bicyclomycin production. The culture of a normal strain was mixed with a typical am$^-$ strain type B in various proportions. The higher the proportion of the am$^-$ strain was in the inoculum, the more depressed was the bicyclomycin production, and the higher was the frequency of am$^-$ colonies. The result obtained with an am$^-$ strain of type C together with a normal strain, as shown in Fig. 11, was the same.

Discussion

Depression of bicyclomycin production in a large scale fermentation raised different problems in its commercial production.

The results of this study indicate that the depression of bicyclomycin production is due to the appearance in the fermentation of abnormal aerial mycelium negative (am$^-$) colonies with a markedly reduced productivity, 1/30 to 1/100 of that of normal strain. The investigations further suggested that the am$^-$ strains are formed as a result of mechanical shock on mycelia caused by the agitation.

Similar phenomena have previously been encountered. In maridomycin fermentation,[3] suppression of growth of *Streptomyces hygroscopicus* has been found to be due to mechanical shock on mycelia caused by glass beads. In *Rhizopus japonicus*,[4] the amount of nucleotides leaking from mycelia was found proportional to the impeller tip velocity. In bicyclomycin fermentation, UV absorbancy of culture filtrates from a culture containing glass beads or from a large scale fermentation was much higher than that of a filtrate of an ordinary flask fermentation (unpublished data).

The results further show that am$^-$ colonies may be formed under other conditions, such as repeated transfers of inoculum, acriflavin treatment of mycelia or higher growth conditions. It has similarly been reported that in streptomycin[5] and novobiocin[6] fermentations, the producing strains degenerated to morphological variants after repeated transfers of inoculum. It has also recently been found[7~10] that the production of antibiotics may be controlled by extrachromosomal elements or plasmids. There has not been any report that plasmids are deleted by mechanical shock on mycelium. However, in bicyclomycin fermentation, simultaneous loss of antibiotic productivity and of aerial mycelium formation by acriflavin treatment or repeated transfer, suggests that production of the antibiotic and aerial mycelium formation in this strain may be controlled by extrachromosomal elements or plasmids.

When shake flasks were inoculated with a mixture of a normal high-producing strain and an am$^-$ low-producing strain, bicyclomycin production was depressed sharply as the proportion of am$^-$ strain in the inoculum increased. At the same time, frequency of the am$^-$ colonies was much higher than could be expected from the initial ratio. Thus, it was evident that the am$^-$ low-producing strain was outgrowing the normal high-producing strain, enhancing the suppression of bicyclomycin production.

It thus appears that in a large scale fermentation of bicyclomycin with agitation, the yield may be markedly reduced because the producing strain by mechanical shock on the mycelia degenerates to an am$^-$, low-producing strain which outgrows the normal strain.

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