EFFECTS OF ETHIDIUM BROMIDE AND ACRIFLAVINE ON STREPTOMYCIN PRODUCTION BY *STREPTOMYCES BIKINIENSIS*

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The treatment of *Streptomyces bikiniensis* with ethidium bromide or acriflavine resulted in the loss of the ability to produce streptomycin in 2~16% of the colonies isolated from the treated spores. These isolates had also lost their resistance to streptomycin. Treatment with the dyes caused partial to total loss of the ability to produce aerial mycelium by *S. bikiniensis*, but the isolates regained this ability upon repeated transfer to fresh medium. The dyes did not appear to effect pigment production by *S. bikiniensis*.

Plasmids have been implicated as determinants of several phenotypic characters in *Streptomyces* species. These include fertility[1,2], melanin[3], aerial mycelium[4,5], antibiotic production[4,6,7,8], and antibiotic resistance[6,7]. The loss of one or more of these characters upon treatment of a streptomycete with acridinium or phenanthridinium dyes has been used to indicate plasmid involvement in the expression of the characters. Acridines, for example, cause the loss of chloramphenicol[4,7], melanin[4], and aerial mycelium[4] production by *Streptomyces venezuelae*. They also cause the loss of kasugamycin and aureothricin production by *Streptomyces kasugaensis*[4] and loss of melanin production and tyrosinase activity in *Streptomyces scabies*[3,4]. Ethidium bromide treatment eliminates the formation of aerial mycelium[5] and loss of antibiotic production (not puromycin) by *Streptomyces alboniger* (B. M. POGELL, personal communication).

Additional evidence for the possible role of plasmids in chloramphenicol and melanin production has been provided by the mapping of the *S. venezuelae* chromosome[7]. The results of these experiments indicated that the character associated with the loss of chloramphenicol production was not located on the chromosomal linkage map.

Recent reports[6,8] also suggest plasmid involvement in the production of methylenomycin A by *Streptomyces coelicolor*. This organism harbors a sex factor termed SCP1[2], and mutations in SCP1 gave progeny that failed to produce the antibiotic. Because antibiotic activity was detected in mixed cultures of these non-antibiotic-producing SCP1+ mutants, the authors concluded that genes that determined steps in the biosynthetic pathway of the antibiotic were located on the plasmid. The factor for resistance to methylenomycin A also appeared to be located on the SCP1 plasmid[8]. Covalently closed circular DNA (M. W. *ca.* 20×10^6 d) has been isolated from *S. coelicolor* A3(2); however, this DNA was found in both SCP1+ and SCP1− strains, so its function is unknown[9].

In this report we describe results of our studies on the effects of ethidium bromide and acriflavine on *Streptomyces bikiniensis*, with emphasis on the possibility of plasmid involvement in streptomycin production.

Materials and Methods

Stock cultures of *S. bikiniensis* (ATCC 11062) and all isolates obtained from that organism were

maintained in sterile soil. Cultures were transferred to Yeast Malt Extract (YMX) agar (Difco Laboratories) for use in the experiments.

Dye Treatment

Spores of *S. bikiniensis* from YMX agar slants were suspended in either 0.05% Triton X-100 or the medium (GCSB) used for growth of *Streptomyces griseus* inoculum[10]. The spores were diluted to a final concentration of approximately 10^3/ml in 10 ml of GCSB medium containing ethidium bromide, 5 μg/ml or acriflavine, 2 μg/ml. Media for control cultures contained no dye. The cultures were allowed to grow on a reciprocal shaker for 5 days following which 0.1 ml aliquots, were withdrawn, transferred to 15-cm Petri plates containing solid GCSB medium (2% agar), and incubated at 26°C for 2~3 days. Individual colonies were then transferred from the plates to YMX agar slants, and the cultures were incubated at 26°C until they had covered the surface of the slant, usually 7~10 days.

Determination of Streptomycin Production

For preliminary screening of the isolates for streptomycin production, plugs of agar were removed from the above slants and transferred to Petri plates containing Antibiotic Medium I (Difco Laboratories) seeded with spores of *Bacillus subtilis* (Difco Laboratories). The plates were incubated at 37°C, and antibiotic production was indicated by a clear zone of inhibition around the agar plugs.

Streptomycin production in liquid medium was determined using the chemically-defined "Lumb's" medium[10]. The *S. bikiniensis* isolates were allowed to grow at 26°C on a reciprocal shaker, and portions of the cultures were removed at intervals and applied to paper assay disks. The disks were placed on assay plates (Antibiotic Medium I) seeded with spores of *B. subtilis*, and streptomycin concentrations were determined by comparison of the diameters of zones of inhibition with a standard curve obtained with known concentrations of streptomycin.

Samples of the culture filtrates from all antibiotic-producing isolates were chromatographed on cellulose thin-layer sheets (Eastman), and their Rf values were compared to streptomycin. The solvent system was 2% *p*-toluene sulfonic acid monohydrate in *n*-butanol saturated with water. Antibiotic activity was detected on the sheets by bioautography with *B. subtilis* as the test organism.

Determination of Streptomycin Sensitivity

A preliminary screening of the isolates for resistance to streptomycin was made by streaking each isolate from control or dye-treated cultures onto solid GCSB medium containing 25 or 50 μg/ml of streptomycin. Isolates that failed to grow at one or both of these concentrations were subjected to more precise sensitivity determinations. These were made by suspending spores and or mycelium in 0.05% Triton X-100 (4 ml) and incorporating the suspensions into 20 ml of solid GCSB medium in Petri plates. The sensitivity of each isolate was determined by applying paper disks containing streptomycin (1~1,000 μg/ml) to duplicate assay plates. After incubation of the plates at 26°C for 24~48 hours, the diameters of the zones of inhibition were measured, and curves plotted from these data were extrapolated back to the diameter of the disks (12.5 mm) to determine the minimum detectable inhibitory concentration.

Production of Pigment and Aerial Mycelium

The production of diffusable pigment by each of the isolates was noted in cultures growing on YMX agar slants and on plates of the tyrosine agar (TA) recommended for the detection of melanin production by *Streptomyces* spp.[11]. Aerial mycelium was observed in the cultures as white, fluffy tufts of hyphae on the upper surfaces of the colonies. Colonies were also examined for the presence of aerial mycelium by low power microscopy.

Results

A preliminary examination for the effects of ethidium bromide and acriflavine on antibiotic production, streptomycin sensitivity, pigment production, and aerial mycelium formation are summarized in Table 1. Two hundred of these isolates from each dye treatment were selected randomly and examined for all four characters. An additional 166 isolates from ethidium bromide treatment and 199 from acriflavine treatment were examined only for antibiotic production. All of the control isolates (ran-

Table 1. Effect of dye treatment on streptomycin production, streptomycin sensitivity, pigment production, and aerial mycelium formation by *S. bikiniensis*.*

Treatment	Streptomycin			Pigment		Aerial Mycelium	
	Production	Sensitivity					
		25 μg/ml	50 μg/ml	YMX	TA	YMX	TA
None	0 (398)	0 (198)	0 (198)	6 (198)	0 (198)	0 (198)	0 (198)
Ethidium bromide	11 (366)	7 (200)	37 (200)	3 (200)	1 (200)	16 (200)	1 (200)
Acriflavine	31 (399)	10 (200)	38 (200)	1 (200)	0 (200)	46 (200)	1 (200)

* Numbers in parentheses indicate total numbers of isolates examined; numbers outside the parentheses indicate the number of isolates that had lost the character indicated at the headings.

Table 2. Distribution of characters lost by *S. bikiniensis* following dye treatment.

Dye	Sm−*	SmR	AM−	Sm−, SmR	Sm−, AM−	Sm− SmR, AM−
Ethidium bromide	0	29	7	5	0	6
Acriflavine	0	8	21	24	0	7

* Sm−, SmR, and AM− are respectively loss of antibiotic production, loss of streptomycin resistance, and loss of aerial mycelium formation.

domly selected isolates from cultures that had not been exposed to dyes) produced detectable antibiotic, and none were inhibited by 25 or 50 μg/ml streptomycin. Eleven of the isolates from ethidium bromide treatment and 31 isolates from acriflavine treatment failed to produce detectable antibiotic activity, and isolates were obtained that were inhibited at streptomycin concentrations of 25 and/or 50 μg/ml. The frequency of loss of antibiotic production varied among experiments from 2~4% with ethidium bromide and from 4~16% with acriflavine. Pigment production by *S. bikiniensis* did not appear to be affected by the dyes but rather seemed to be related to culture medium. Only one isolate, which was obtained by ethidium bromide treatment, failed to produce pigment on the TA medium, but that isolate produced pigment when grown on YMX agar. Sixteen cultures isolated after ethidium bromide treatment and 46 isolates from acriflavine treatment produced little or no aerial mycelium when allowed to grow on YMX agar. Only one isolate from each dye treatment failed to form aerial mycelium on both YMX agar and TA medium.

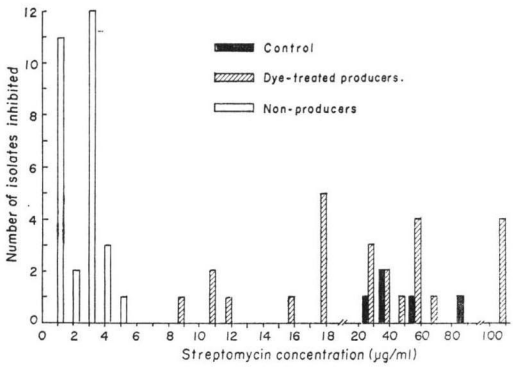
Of the 765 cultures examined after dye treatment, 137 appeared upon preliminary examination to have lost one or more of the following properties: antibiotic formation, streptomycin resistance, or aerial mycelium formation (Table 2). The data in Table 2 were obtained from the 200 isolates that were chosen randomly and from those that were retained because of their inability to produce antibiotic. All three characters appeared to have been lost by 13 of the isolates, two characters by 29 isolates, and one of the characters by 65 isolates. An important correlation seen in Table 2 is that all of the isolates that had lost the ability to produce antibiotic had also become sensitive to streptomycin at concentrations of 25 and/or 50 μg/ml.

Fifty-nine of the 137 isolates that had lost one or more of the above characters were chosen for further study. The production of antibiotic by the isolates was examined in the synthetic, liquid medium. All of the isolates that failed to produce streptomycin when grown on YMX agar in the preliminary

experiments also were unable to produce antibiotic on this medium. All isolates that produced antibiotic on the agar medium also produced on the liquid medium; however, the maximum amounts varied from 13 μg/ml to greater than 1,000 μg/ml (as measured by streptomycin standards). Antibiotic activity could be detected after 48 hours growth in most cultures, but a few of the isolates did not begin producing until 72 hours and one not until 96 hours. Cellulose thin-layer chromatography of these antibiotic-producing cultures and comparison with streptomycin standards indicated that the only antibiotic in the culture fluids was streptomycin.

A comparison of the sensitivity of the isolates to streptomycin is shown in Fig. 1. Minimum detectable inhibition of the controls was observed at streptomycin concentrations of 20~90 μg/ml. The minimum inhibitory concentration for the streptomycin-producing cultures isolated after dye treatment varied from 9 to nearly 200 μg/ml. In contrast, growth of the non-streptomycin-producing isolates was inhibited at streptomycin concentrations of 5 μg/ml or less. Streptomycin production by the producing isolates did not appear to be related to the sensitivity of the isolate to the antibiotic. For example, the most sensitive producing isolate (inhibited by 9 μg/ml) produced a maximum of 125 μg/ml of streptomycin, and another isolate that produced more than 1,000 μg of streptomycin per ml was inhibited by 18 μg/ml.

Fig. 1. Sensitivity of *S. bikiniensis* isolates to streptomycin.



The ability of the 59 isolates to regain lost characters was examined by repeated transfers to fresh YMX agar medium or to soil. None of the isolates that had lost the ability to produce streptomycin and also had lost resistance to streptomycin regained either of these properties after as many as 6 transfers; thus the loss of these characters appears to be stable.

In contrast, the loss of the ability to form aerial mycelium was an unstable character. While some of the cultures formed no aerial mycelium on their first transfer to slants, other isolates had a few colonies that were heavily tufted and still other isolates had colonies all of which were partially covered with aerial mycelium. All of these isolates regained the ability to form aerial mycelium and spores after one or two transfers to fresh YMX agar medium. Although a few of the isolates did not form aerial mycelium as long as they were maintained in sterile soil, these also formed aerial mycelium after transfer to YMX agar.

Discussion

The data presented here indicate that treatment with ethidium bromide or acriflavine caused the high frequency loss of antibiotic production by *S. bikiniensis*. The identity of the antibiotic was confirmed by thin-layer chromatography, and this identification was supported by the observation that antibiotic-producing isolates of *S. bikiniensis* were resistant to streptomycin. The loss of streptomycin production was always accompanied by a decreased resistance to streptomycin. Growth inhibition of most non-producers occurred at streptomycin concentrations of 1~4 μg/ml, about the same levels that inhibit growth of *B. subtilis*. None of the non-producing isolates regained either the ability to produce streptomycin or acquired resistance to that antibiotic through repeated transfer of the cultures.

There was a wide variation in sensitivity to the antibiotic among the different streptomycin-producing isolates. The presence of a few producing isolates (Fig. 1) that are sensitive to streptomycin at concentrations between 9 and 18 μg/ml suggests that dye treatment might have caused partial loss of resistance in these isolates. This interpretation is questionable, however, because 23 of the 30 producing isolates examined are capable of producing streptomycin in amounts exceeding their apparent sensitivities. It appears more likely that our results reflect a variation in sensitivity of the isolates to streptomycin during early stages of growth. As discussed by DEMAIN[12], many organisms develop enhanced resistance to their own antibiotics during or just prior to the phase in which the antibiotic is being synthesized. In *S. bikiniensis* cultures, as in cultures of many other *Streptomyces* species, antibiotic production occurs after growth of the cultures has nearly ceased (SHAW and PIWOWARSKI, unpublished results).

Neither ethidium bromide nor acriflavine appeared to have any effect on production of the brown-black pigment by *S. bikiniensis* on YMX agar on TA. Acridine dyes cause the loss of melanin pigment production by *S. scabies*[3]; however, the pigment produced by *S. bikiniensis* on TA probably is not melanin[13]. The dyes appeared to cause the loss of aerial mycelium formation by *S. bikiniensis* as with *S. alboniger*[5], but the loss was not always complete nor was it permanent.

The mechanism by which ethidium bromide and acriflavine induce apparently permanent changes in *S. bikiniensis* is unknown, but the known action of these dyes[14,15] suggest the possible involvement of a plasmid(s) in the production of streptomycin and in resistance to that antibiotic. Other studies are in progress to test this hypothesis. Our results do not indicate the involvement of plasmids in pigment production or in aerial mycelium formation by *S. bikiniensis*, but they do not rule out such involvement.

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