The Production of Streptomycin by *Streptomyces bikiniensis*

Donald B. Johnstone and Selman A. Waksman

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THE PRODUCTION OF STREPTOMYCIN BY STREPTOMYCES BIKINIENSIS[1, 2]

DONALD B. JOHNSTONE AND SELMAN A. WAKSMAN

New Jersey Agricultural Experiment Station, New Brunswick, New Jersey

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Among the various antibiotics produced by actinomycetes, streptomycin occupies a prominent place. So far, the ability to form this substance has been limited to certain strains of organisms belonging to the *Streptomyces griseus* group (Schatz, Bugie, and Waksman, 1944; Waksman, Reilly, and Johnstone, 1946). Most of the other strains of *S. griseus* are unable to produce any antibiotic at all (Waksman, Schatz, and Reynolds, 1946; Carvajal, 1946), or they form other antibiotics, such as grisein (Reynolds, Schatz, and Waksman, 1947). The streptomycin-producing strains of *S. griseus* can easily be differentiated from the nonstreptomycin strains. Two simple procedures can be utilized for this purpose: (1) determination of the sensitivity of the streptomycin strains and the resistance of the nonstreptomycin strains to the action of actinophage (Waksman, Harris, and Reilly, 1948); (2) determination of the resistance of the first and the sensitivity of the second to the action of streptomycin. Sensitivity to streptomycin can be determined by the agar streak method, streaking first a streptomycin-producing strain of *S. griseus* and, after 24 hours' incubation, streaking the unknown strains as test organisms.

The possibility that streptomycin may be produced by organisms other than *S. griseus* has been indicated recently by Trussell, Fulton, and Grant (1947), who isolated a culture of a *Streptomyces* which produced a mixture of antibiotics; one of these appeared to be streptomycin and the other streptothricin. Johnstone and Waksman reported (1947) that a certain organism, tentatively designated as *Streptomyces bikiniensis* and distinct from *S. griseus*, was capable of elaborating an antibiotic that proved to be very similar to streptomycin. Since the chemical identity of this substance with the streptomycin obtained from *S. griseus* was not fully established, the preparation was designated tentatively as streptomycin II. The experimental results dealing with the nature of the organism and with its ability to produce streptomycin form the subject of this paper.

EXPERIMENTAL PROCEDURES AND RESULTS

Isolation of organism. In connection with a study of the bacteriological activities in the waters of the Bikini Lagoon and of neighboring areas, carried out during the atomic bomb experiments in July, 1946, several soil samples were taken from the Bikini and Rongelap atolls. The location of these soils, their

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chemical composition, and their biological properties were reported elsewhere (Johnstone, 1947).

When brought to the laboratory, these soils were plated out, ordinary bacteriological media being used. After certain periods of incubation at 28 C, the plates were examined for the nature and abundance of the various groups of organisms. One of the most striking properties of the microbiological population of those soils was the relatively high percentage of actinomycetes among the colonies developing on the plates. The actinomycetes colonies were picked from the plates and tested for their antagonistic properties by the usual agar cross-streak method. A large number of the cultures, belonging mostly to the genus *Streptomyces*, exerted an inhibiting effect upon the growth of the test bacteria on the agar plate. One of the cultures showed marked inhibition of growth of various gram-positive and gram-negative bacteria. When grown in liquid media, this culture produced an antibiotic that resembled streptomycin in its

TABLE 1

Inhibition of bacterial growth by actinomycetes as measured by cross-streak tests

STREPTOMYCES SPECIES	TEST BACTERIA			
	Bacillus subtilis	*Escherichia coli*	*Mycobacterium avium*	*Mycobacterium phlei*
	Zone of inhibition, in millimeters			
S. bikiniensis	30	21	26	30
S. griseus 4	20	15	13	19
S. griseus 22	20	10	14	19
S. lavendulae	25	20	20	20
Streptomyces G	8	10	14	20
Streptomyces H	20	9	21	22

antibacterial properties or its antibiotic spectrum, in its solubility in water and other solvents, and in various other chemical and biological reactions. Since the culture was, however, distinctly different from *S. griseus*, it was selected for further study. Since it was also different from the other known species of *Streptomyces*, it was decided to designate it as a new species, under the name *Streptomyces bikiniensis*.

The results obtained by the cross-streak agar method (table 1) established the degree of inhibition of various bacteria by *S. bikiniensis*, as compared to similar inhibition of the same bacteria by two strains of streptomycin-producing *S. griseus*, the streptothricin-producing *Streptomyces lavendulae*, and two unknown cultures belonging to the genus *Streptomyces*. *S. bikiniensis* gave on the plate a wider zone of inhibition against the various test bacteria than did the other actinomycetes. When a more complete antibacterial spectrum was made, comparing the relative inhibition of the growth of a number of bacteria, *S. bikiniensis* was found to give a spectrum similar to that of the two strains of *S. griseus*; it was quite distinct, however, from the spectra of the other three actinomycetes.

Production of streptomycin II. *S. bikiniensis* was grown in the same broth that is commonly used for the production of streptomycin. This broth consists of 3 g meat extract, 5 g peptone, 10 g glucose, and 5 g NaCl per liter of tap water. The cultures were incubated at 28 C, both under static and shaken conditions. Some of the cultures were removed after definite incubation periods, filtered through paper, and the filtrates tested by the agar streak method (Waksman and Reilly, 1945). The results presented in table 2 show that the static cultures were somewhat more satisfactory than were the shaken cultures for the growth of *S. bikiniensis* and for the production of the antibiotic. Although the shaken cultures gave greater activity in a shorter time, this activity never became so high as in the static cultures and soon tended to disappear completely. The

TABLE 2

Production of an antibiotic substance by Streptomyces bikiniensis

INCUBATION	B. SUBTILIS	B. MYCOIDES	E. COLI	S. MARCESCENS
		Static cultures		
days				
3	30	10	0	0
6	300	100	10	10
10	300	300	30	30
14	200	200	30	30
		Shaken cultures		
1	100	0	0	0
3	100	30	10	10
5	100	100	30	30
7	10	0	0	0

Dilution units per 1 ml of culture.

antibacterial spectrum of the culture filtrates appeared in both cases, however, to be very similar to that of streptomycin.

The effect of the nature of the various constituents of the medium and of their concentration upon the production of the antibiotic, *S. bikiniensis* was studied in shaken cultures. The cup method, with a streptomycin standard, was used for testing the activity of the cultures. In the case of glucose, the highest activity was obtained with 0.1 per cent of the sugar, as shown in figure 1. The cultures free from glucose gave as good activity as those containing the higher sugar concentrations. With 2.0 per cent of glucose, the medium remained acid for a long time. This initial acidity was later overcome by the growing culture, the pH rising to its stabilized peak of 8.7. This peak was reached sooner in cultures containing smaller amounts of glucose. The amount of streptomycin produced in 9 days with 2 per cent glucose was only 20 μg per ml, thus showing that glucose has a tendency to delay the formation of streptomycin. Apparently the buffering effect of the glucose, which was found to be necessary in *S. griseus*

cultures, is not essential in cultures of *S. bikiniensis*, the highest titers having been obtained in the cultures containing only 0.1 per cent glucose. The pH pattern was the same with the small amount of glucose as with the glucose-free culture.

Among the other constituents of the medium, sodium chloride was found to be most effective in 1 per cent concentration. Peptone proved to be a better nitrogen source than tryptone, tryptose, or proteose. Meat extract exerted but

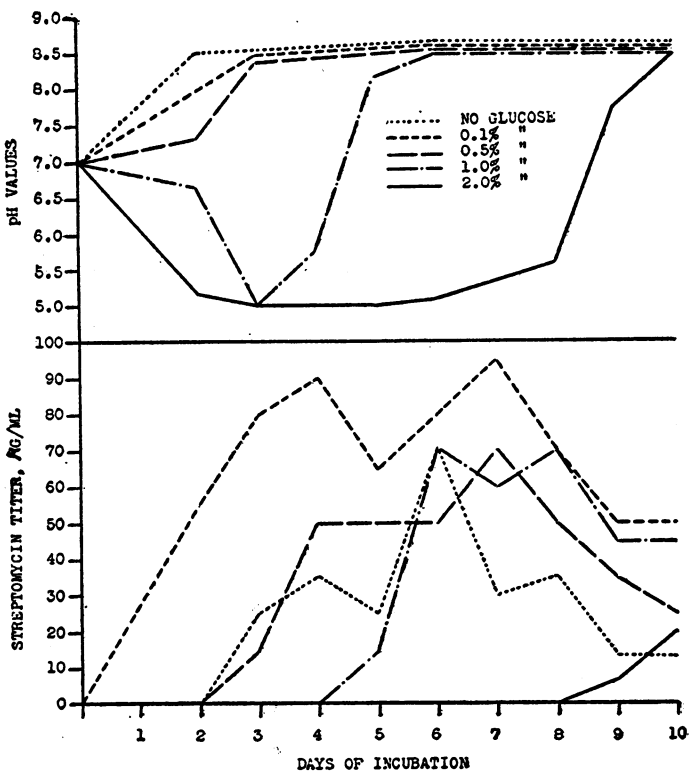

FIG. 1. INFLUENCE OF DIFFERENT CONCENTRATIONS OF GLUCOSE UPON CHANGES IN REACTION AND PRODUCTION OF STREPTOMYCIN BY S. BIKINIENSIS IN SUBMERGED CULTURE

little effect upon the production of the antibiotic. This was particularly surprising, since meat extract or its equivalent, such as corn steep or soybean meal, is usually required for the production of streptomycin by *S. griseus*, although synthetic media were recently found to give good yields of this antibiotic. The addition of $FeSO_4$ to the medium had no effect upon the production of streptomycin; $ZnSO_4$, however, in concentrations of 50 mg per liter, produced a marked stimulating effect. When both ions were used, the presence of iron tended to neutralize the favorable effect of the zinc.

Isolation of streptomycin II. The antibiotic produced by *S. bikiniensis* was isolated from the culture medium by the method commonly employed for the

isolation of streptomycin. The active material was readily removed from the culture filtrate by adsorption on norit A. The adsorbate was washed with neutral alcohol and treated with acidified ethyl alcohol. The alcoholic extract was filtered, neutralized, and the alcohol driven off by distillation at low temperatures. The residue was dried *in vacuo*. The active material could be precipitated directly from the alcoholic solution by the addition of several volumes of acetone. It could also be isolated by various procedures commonly used in the isolation of streptomycin, such as the methyl alcohol formic acid method. Preparations

TABLE 3

*Antibiotic spectra of streptomycin and streptomycin II**

TEST ORGANISM	STREPTOMYCIN	STREPTOMYCIN II
Gram-positive bacteria		
B. subtilis	0.3	0.3
B. mycoides	1.0	1.0
B. megatherium	1.5	1.5
B. circulans	0.3	0.2
B. cereus	5.0	5.0
S. aureus	5.0	5.0
S. lutea	0.1	0.1
M. lysodeikticus	1.0	1.0
M. avium	0.5	0.5
M. phlei	0.05	0.05
M. tuberculosis 607	0.3	0.3
Gram-negative bacteria		
E. coli	1.0	1.0
S. marcescens	3.0	5.0
S. alkalescens	9.0	5.0
S. paradysenteriae	10.0	10.0
S. dysenteriae	5.0	5.0
A. aerogenes	3.0	3.0

* Expressed as micrograms of streptomycin required to inhibit the growth of the organisms in 1 ml of glucose-free nutrient broth.

were thus obtained that had an activity of 30 to 50 units per milligram, comparable to the streptomycin yields obtained by similar procedures in the early studies with *S. griseus*.

The *S. bikiniensis* culture was plated out on suitable media, and individual colonies were picked and tested. Some of the strains thus obtained were more active in producing the antibiotic than was the original culture. By using these isolated strains and improving the method of extraction, preparations were obtained that assayed as high as 158 units per milligram against a streptomycin standard.

The streptomycin II preparations were compared to the regular streptomycin for their respective antimicrobial properties. The results were reported (table 3) on the basis of micrograms of streptomycin required to inhibit the growth of the

various test organisms. The two antibiotic spectra are identical. In the case of *M. tuberculosis* H37, the amount required for growth inhibition was exactly 2.1 g per ml for both streptomycin and streptomycin II, as determined by the turbidimetric method (Smith, 1947). These findings were obtained on several repeated tests. The streptomycin-resistant strain of *M. tuberculosis* H37Rv also proved to be resistant to streptomycin II.

In addition to these identical antibacterial spectra, the two preparations showed the following similarities in their action upon other organisms: (1) Both forms of streptomycin were inactive against fungi. (2) Bacteria made resistant to streptomycin were also resistant to streptomycin II. (3) Strep-

TABLE 4

Comparative effects of streptomycin and streptomycin II upon Staphylococcus aureus infection in mice

NO. OF MICE	PREPARATION	UNITS PER MOUSE	CULTURE DILUTION	NUMBER OF MICE ALIVE AFTER DAYS				
				1	3	5	7	10
10	Controls	—	10^{-3}	0	—	—	—	—
10	"	—	10^{-4}	2	2	1	1	1
10	"	—	10^{-5}	4	1	—	—	—
10	Streptomycin	5	10^{-3}	1	1	1	1	1
10	"	10	"	3	3	3	3	3
10	"	25	"	9	6	6	6	6
10	"	50	"	10	10	10	10	10
10	"	100	"	10	10	10	10	10
10	Streptomycin II	5	"	1	1	1	1	1
10	" "	10	"	3	2	2	2	2
10	" "	25	"	9	6	6	6	6
10	" "	50	"	10	10	10	10	10
10	" "	100	"	10	10	10	10	10

Culture, *S. aureus* SM. in 4 per cent mucin; mode of drug administration, subcutaneous; duration of therapy, single dose immediately after infection.

tomycin II was inactivated by cysteine, in a manner similar to the inactivation of streptomycin. (4) Both forms of streptomycin gave the same type of reduced activity in the presence of glucose. (5) Both preparations were equally sensitive to increased acidity of the medium.

Toxicity and in vivo activity of streptomycin II. Preliminary toxicity tests with streptomycin II, using chick embryos, demonstrated that this preparation when given in large doses—more than 1,200 units per embryo—could be administered with 100 per cent survival.

Samples of the preparation were submitted to the Merck Institute for detailed *in vivo* studies. The results obtained by them emphasized further a remarkable identity in the behavior of streptomycin II with streptomycin.

By courtesy of the Institute, the results of a typical experiment are reported in table 4. Similar effects were obtained when both preparations were used against *Salmonella schottmülleri* in mice.

These results thus prove definitely that *S. bikiniensis*, an organism that belongs to the actinomycetes and that was isolated from a Bikini soil, an organism distinct, both morphologically and culturally, from *S. griseus*, produces an antibiotic that is similar to, if not identical with, streptomycin. Streptomycin II and streptomycin exhibit comparable antibiotic spectra, similarity in physical and chemical properties, and low toxicity to animals. Until the new antibiotic has been crystallized and its clinical activity determined, its absolute identity with streptomycin cannot be established. In view of this, as well as of the minor quantitative differences in the respective antibiotic spectra, and especially of differences in the nature of the organisms producing the two antibiotics, the designation of the newly isolated antibiotic as streptomycin II, namely a streptomycinlike substance, may still be preserved.

Description of Streptomyces bikiniensis

Morphology. On glucose asparagine agar, the aerial mycelium arises from the agar surface in the form of single hyphae that subsequently branch heterogeneously. As sporulation develops, these branched mycelia bear straight chains of oval conidia. No tendency to form spirals was noted (figure 2).

Synthetic agar (Czapek's). Growth is luxuriant, white, becoming pallid neutral gray (Rdg.[3] LIIING-f) with white tinge. Aerial mycelium and spores formed abundantly. Superficial droplets, amber-colored. Soluble pigment, light brown.

Glucose asparagine agar. Growth luxuriant with good aerial mycelium and spores white, becoming light mouse-gray (Rdg. LI 15 ′′′′′ Y-O-b). White area in center of slant base remains for about a week, and white fringe remains for a longer period. Superficial droplets, colorless. Soluble pigment, very light amber to no pigment at all.

Nutrient agar. Growth luxuriant with moderate aerial mycelium. Color remains white with scanty sporulation. Superficial droplets, none. Soluble pigment, deep brown.

Nutrient agar plus glucose. Growth similar to that on nutrient agar, but gray color and spores finally produced. Superficial droplets, amber-colored. Soluble pigment, deep brown, darker than that in nutrient agar.

Potato plug. Growth raised and wrinkled; color, pale ochraceous-buff (Rdg. VV 15′f), localized and not spreading. Plug, dark brown, local area adjacent to growth, almost black.

Gelatin. Liquefaction slight, 1 cm per week at 37 C.

Nutrient broth. Growth abundant, complete white surface pellicle formed. Superficial droplets, none. Soluble pigment, deep brown.

[3] Rdg. = R. Ridgway, Color standards and color nomenclature, Washington, D. C., 1912.

Nutrient broth plus glucose. Growth abundant, complete white surface pellicle formed. Aerial mycelium becomes gray in patches. Superficial droplets, amber-colored. Soluble pigment, deep brown.

Milk. Reaction distinctly alkaline. Coagulation, none. Hydrolysis takes place in 5 days at 28 C. Growth patchy, surface growth of white aerial mycelium becoming gray with sporulation.

Starch. Growth abundant, white aerial mycelium becoming gray with sporulation. Hydrolysis slight, observable after 3 days at 28 C.

Habitat. Organisms isolated from a soil obtained from the island of Bikini, Bikini Atoll, in the northern Marshall Islands, during the Bikini bomb experiments in July, 1946. Soil pH 9.0.

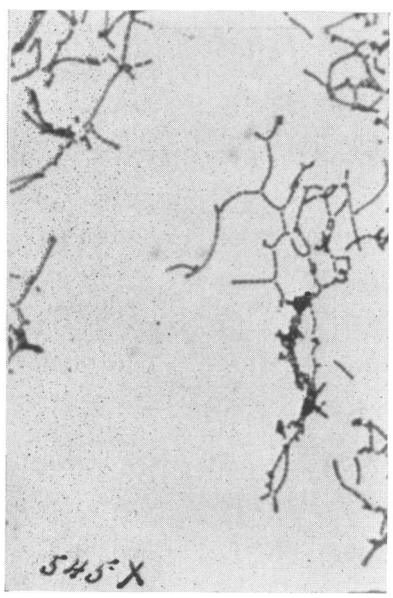

FIG. 2. SPORULATION OF STREPTOMYCES BIKINIENSIS

Comparison to Related Species

The following discussion of related species of the genus *Streptomyces* is based upon a comparison with the earlier work carried out in this laboratory on soil actinomycetes (Waksman, 1919).

S. bikiniensis is similar to *Streptomyces aureus* in some of its cultural properties; both produce a gray-colored aerial mycelium on some of the media. On other media, however, even this property is different: *S. aureus* produces a white mycelium on nutrient agar and a cream-colored mycelium on glucose-containing agar, whereas the corresponding pigmentation of the mycelium of *S. bikiniensis* is white and gray. The sporulation of *S. aureus* takes place in the form of spirals in its aerial mycelium, whereas *S. bikiniensis* is completely devoid of spirals in its mycelium on all the media tested, as shown in figure 2.

Streptomyces olivochromogenus possesses cultural characteristics that are similar to those of the new culture, with the exception of its growth on potato plug. *S. olivochromogenus* also produces closed spirals, whereas *S. bikiniensis* does not.

S. bikiniensis resembles *Streptomyces* no. 145 of Waksman (1919) in that it is devoid of spirals and produces gray aerial mycelium on glucose-asparagine agar. The latter organism, however, produces gray aerial mycelium and forms no soluble brown pigment on nutrient agar, both of which are contrary to the characteristic properties of *S. bikiniensis*.

S. bikiniensis resembles most closely *Streptomyces griseolus* Waksman. Both cultures are similar culturally and morphologically, as shown by a lack of spiral formation. The only difference between the two cultures was noted in their growth on potato plug: *S. griseolus* overgrows the plug and produces greenish aerial mycelium, whereas *S. bikiniensis* is limited to localized nonspreading growth on the plug, the color of the growth remaining buff. In addition, the pigmentation of the two cultures on nutrient agar is different; there are also differences in the nature of their growth on glucose asparagine agar, as shown by the following summary:

ORGANISM	GLUCOSE-ASPARAGINE AGAR	POTATO PLUG
S. bikiniensis	Surface growth velvety, white border; gray aerial mycelium with colorless droplets of water on surface. Vegetative mycelium white.	Buff to gray-colored growth, not spreading. Plug pigmented black.
S. griseolus	Surface growth thin, dry; gray aerial mycelium with no water droplets. Vegetative mycelium dark in color.	Raised growth, buff to gray in color, spreads over whole surface of plug. Plug not pigmented.

Because of these cultural differences, because of the specific nature of the substrate from which the organism was isolated, and especially because of the specific physiological properties of the organism producing streptomycin, the authors felt justified in designating this organism as a new species, under the name of *Streptomyces bikiniensis*.

SUMMARY

A culture of an actinomycete was isolated from a Bikini soil. This culture grown in an artificial medium, namely, in meat-extract-peptone-glucose broth, produced an antibiotic substance that appeared to be identical with streptomycin. The identity has been established by similar antimicrobial spectra, similar action upon resistant strains of bacteria, similar reactivity with various chemical reagents, and similar effects upon experimental animals. The new streptomycin preparation, however, has not been purified chemically and, as long as its chemical identity with streptomycin has not been established, it has been tentatively designated as streptomycin II, a streptomycinlike antibiotic.

A study of the specific nature of the organism producing streptomycin II

showed it to be markedly different from *Streptomyces griseus* in its morphological and cultural characteristics. It is somewhat similar to *Streptomyces aureus*, to *Streptomyces olivochromogenus*, and especially to *Streptomyces griseolus*. However, it is not identical with any one of them. Because of this, because of the specific substrate from which it was isolated, and because of characteristic physiological properties that result in the production of streptomycin, it is described as a new species, *Streptomyces bikiniensis*.

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