A New Hybrid Strain of an Oxytetracycline-producing Organism, *Streptomyces rimosus*

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Abstract

Mindlin, S. Z. (All-Union Research Institute for Antibiotics, Moscow, U. S. S. R.), S. I. Alikhanian, A. V. Vladimirov, and G. R. Mikhailova. A new hybrid strain of an oxytetracycline-producing organism, *Streptomyces rimosus*. Appl. Microbiol. **9:** 349–353. 1961.—A new strain, *Streptomyces rimosus* LS-T hybrid, characterized by a lower level of foaming and higher antibiotic activity as compared to the other active strains of *S. rimosus*, is obtained as a result of selection among prototrophic recombinant forms of strains LS-T293 and BS-21. The studies on strain LS-T hybrid show the possibilities of combining properties of different strains of *S. rimosus* in the hybrid forms. The occurrence of new properties in the hybrid forms is detected.

Hybridization is one of the basic methods used in the breeding of higher plants and animals. Up to the present time, the use of this method in selection of microorganisms, excluding yeasts and some eumycophyta, has been very limited, as microorganisms were thought to have no sexual reproduction. Recently, however, this question was revised as processes roughly similar to sexual reproduction were observed in a series of microorganisms, including those used in industry. The possibility of obtaining heterozygous diploids in penicillia and aspergilli was shown, and genetic recombinations in various species of actinomycetes were obtained (Pontecorvo, 1956; Sermonti and Spada-Sermonti, 1956). This finding suggested the selection of strains which combined the features of different parent forms, and the use of hybridization methods in selection of microorganisms became practicable.

The possibility of obtaining recombinants in a strain of *Streptomyces rimosus* producing the broad spectrum antibiotic, oxytetracycline, was shown by us earlier (Alikhanian and Mindlin, 1957). The crossing of single biochemical mutants yielded several types of prototrophic forms which differed (besides other properties) in their ability to synthesize the antibiotic. Hybrid forms, equal by their antibiotic activity to the starting strains and sometimes even exceeding them in this respect by 10 to 15%, were observed in almost all combinations studied. By contrast, the corresponding biochemical nutritional mutants were very poor producers of the antibiotic (Alikhanian and Mindlin, 1958; Alikhanian, 1959*a*). In these experiments, one more interesting fact in favor of using hybrid prototrophic forms of *S. rimosus* in selection of new strains was observed. It was found that prototrophic recombinants obtained as a result of mixed growth of two biochemical mutants possessed new physiological properties differing from those of the starting strains.

On the basis of these data we attempted to obtain a new highly productive strain of *S. rimosus* through selection of hybrid forms. Our task was not only to increase the activity of the present strains, but also to obtain a strain suitable for fermenting concentrated media, as all highly active strains available caused great foaming of the fermentation fluid and could not be used for cultivation in these conditions.

The obtaining of such a strain is described here.

Materials and Methods

Two strains of *S. rimosus* differing in their origin were used. Strain LS-T293 was obtained by us after selection among ultraviolet-induced variants of *S. rimosus* (Alikhanian, 1959*b*). It is characterized by producing high levels of oxytetracycline in media containing higher concentrations of inorganic, soluble phosphorus as compared to other strains of *S. rimosus*. Strain BS21 was received from Hungary. It is inferior in its activity to strain LS-T293 and does not require higher levels of phosphorus in the media. In addition, both strains differ by a number of morphological and physiological properties.

For obtaining hybrid forms from these strains, their biochemical mutants were used. Biochemical mutants of these strains were as follows: variant no. 80 requiring methionine and variant no. 1 requiring valine and isoleucine were obtained from strain LS-T293; and variant no. 49 requiring histidine and variant no. 13 requiring leucine were obtained from strain BS-21. These biochemical mutants served as the starting material in our work.

The hybrid forms were obtained by a usual procedure (Sermonti and Spada-Sermonti, 1956). Spores from a mixed culture of the biochemical mutants were plated on selective media and the prototrophic colonies grown were picked.

To study the antibiotic activity of different strains they were grown at 26 to 28 C in flasks on a rotary shaker at 220 to 240 rev/min. Two media were used for the cultivation of the strains; medium A containing 0.25 to 0.75% of corn steep liquor and 3% of starch and medium B, a concentrated medium, containing 0.5% of corn steep liquor, 2% of soybean meal, and 5% of starch.

The antibiotic activity was determined by a photocolorimetric method. The foaming level was determined visually by presence or absence of foam in the fermentation flasks.

Results and Discussion

At the first stage of our work, prototrophic hybrid forms were obtained from a mixed growth of biochemical mutants of strains LS-T293 (nos. 80 and 1) and BS-21 (nos. 49 and 13), in combinations 49 × 1, 49 × 80, and 13 × 1.

The second stage of our work was the selection of a prototroph which would most closely meet our requirements. The antibiotic activity and the level of foaming were the two properties of the prototroph evaluated. The results of this work are presented in Table 1. As a control, a highly active strain LS-T118 (Mindlin and Alikhanian, 1958) was used. As is evident from Table 1, two recombinant strains causing practically no foaming of the fermentation media were obtained, that is a hybrid of type I from crossing mutant no. 13 and mutant no. 1 (13 × 1) and a hybrid of type I from crossing 49 × 80. As far as their antibiotic activity is concerned, only one of these strains could compete in this respect with strain LS-T118. This was the prototrophic recombinant of type I from combination 49 × 80, subsequently named strain LS-T hybrid.

TABLE 1. *Data on evaluation of the antibiotic activities and the levels of foaming produced by hybrid forms of Streptomyces rimosus mutants BS-21-no. 49 and LS-T293-no. 1*

Strain	Activity of new strain as percentage of the activity of strain LS-T118	Evaluation of the foam levels	
		Total no. of fermentations	The no. of fermentations with no foaming
Strain LS-T118 (control)	100	7	1
Hybrids from combination 49 × 1:			
Type I	90–95	4	3
Type II	100	2	1
Type III	70	1	0
Hybrids from combination 13 × 1:			
Type I	60–66	5	5
Type II	90–93	2	1
Type III	100–110	2	1
Hybrids from combination 49 × 80:			
Type I	100–103	7	7
Type II	80	2	1

A detailed study of this strain also showed its superiority to strain LS-T118 with respect to the amount of antibiotic produced. The new strain exceeded strain LS-T118 in its activity by 10 to 15%. The comparative data on the antibiotic activities of these strains, grown on concentrated media in laboratory conditions, are presented in Table 2.

Because of these properties, strain LS-T hybrid was tested on an industrial scale.

The task of obtaining a new strain, superior in its antibiotic activity to the highly active strain and

TABLE 2. *Comparative antibiotic activities of Streptomyces rimosus strains LS-T118 and LS-T hybrid type I from crossing 49 × 80*

Strain	No. of flasks	Oxytetracycline activity	
		μg/ml	As percentage of the activity of strain LS-T118
LS-T118	43	4,353	100
LS-T Hybrid (type I)	44	4,909	113

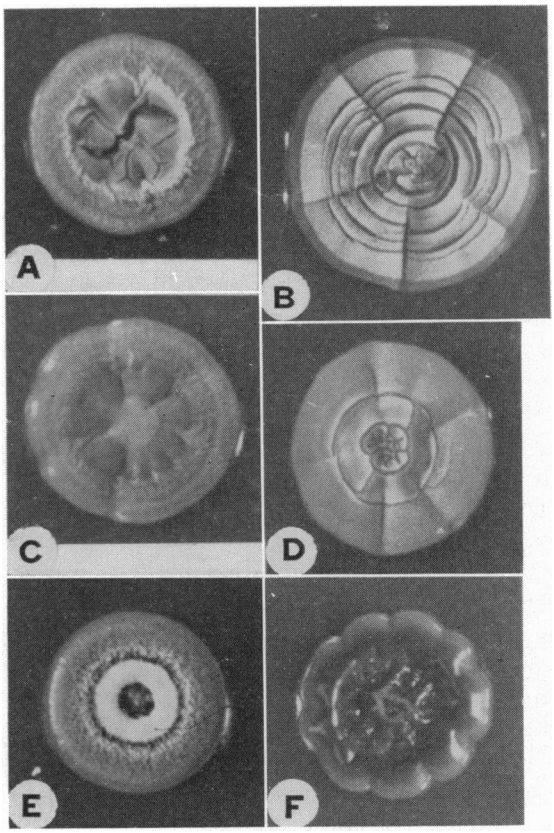

FIG. 1. *Colonies of the starting strains, their biochemical mutants, and the hybrids, obtained as a result of their crossing on the corn steep liquor medium. (A) Strain LS-T293, (B) Strain BS-21, (C) biochemical mutant no. 80 requiring methionine, (D) biochemical mutant no. 49, requiring histidine, (E) hybrid of type I,* (F) *hybrid of type II.*

characterized by a lower level of foaming, was solved without significant difficulties.

However, as in other cases mentioned above, the crossing of biochemical mutants resulted in the occurrence of forms characterized by new physiological properties differing from those of the initial strains. Thus, both starting strains, LS-T293 and BS-21, caused great foaming of the fermentation media, whereas the hybrid strain markedly differed from them in this respect. In this connection it was interesting to trace

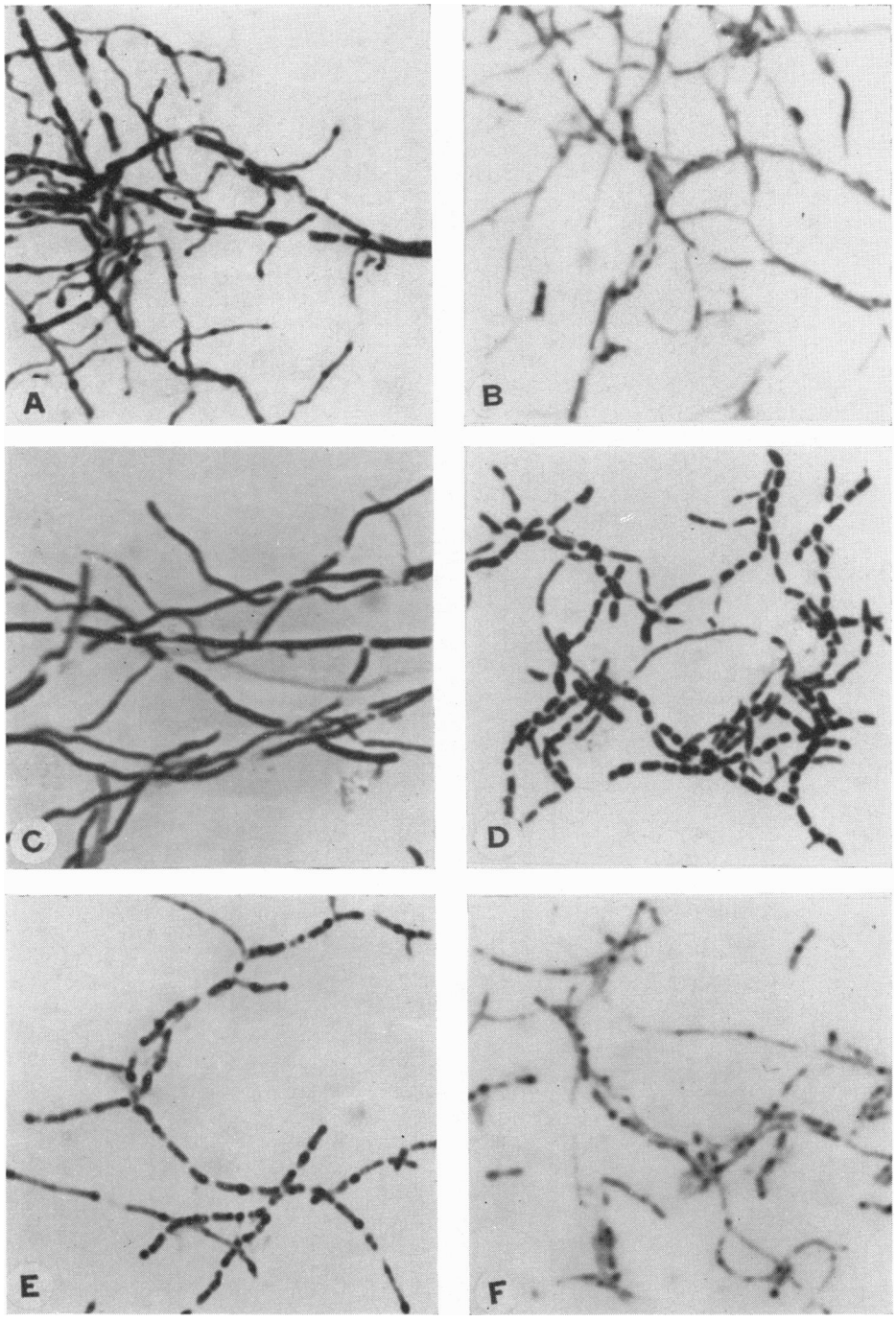

FIG. 2. *Mycelia of strains LS-T293, BS-21, and the hybrid of type I (LS-T hybrid) fixed with Carnoy fluid and stained with methylene blue after 24 and 48 hr of their growth in submerged culture. Magnification 2,000× (microscope MBI-3, microphoto contrivance FMN-3, ocular K10×, apochromatic objective ×90). (A) basophilic branched hyphae, containing volutin (strain LS-T293, 24 hours); (B) low basophilic, thin hyphae, containing single small volutin granules, and partially germinating fragments of thick, basophilic hyphae (strain LS-T293, 48 hr); (C) high basophilic, thick hyphae (strain BS-21, 24 hr) (D) partially germinating fragment chains of high basophilic hyphae (strain BS-21, 48 hr); (E) basophilic slightly branched hyphae (hybrid of type I, 24 hr); (F) low basophilic, thin hyphae and the fragments of thicker, basophilic hyphae, containing volutin (hybrid of type I, 48 hr).*

the inheritance of other features from strains LS-T293 and BS-21 by strain LS-T hybrid and one more prototrophic recombinant from crossing 49 × 80.

As mentioned above, strains LS-T293 and BS-21 have many differences. Strain LS-T293 possesses higher activity than strain BS-21. It is more sensitive to X irradiation and exhibits some sensitivity to low concentrations of streptomycin, whereas strain BS-21 is resistant to streptomycin even at the concentrations of 1,000 μg/ml. These strains differ by their cultural

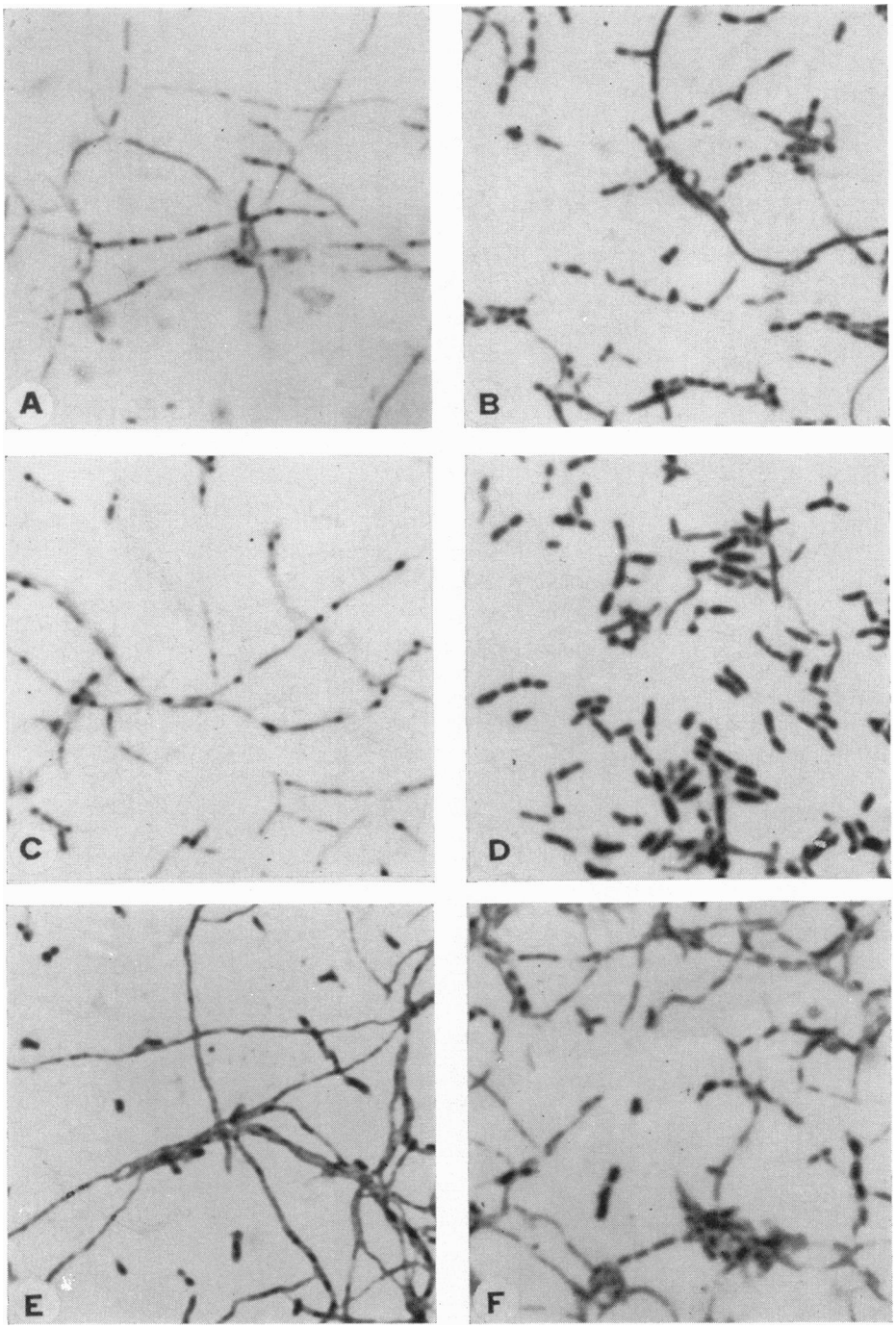

FIG. 3. *Mycelia of the starting strains, their biochemical mutants, and the hybrids, obtained as a result of their crossing, fixed with Carnoy fluid and stained with methylene blue after 72 hr of their growth in submerged culture. Magnification 2,000× (microscope MBI-3, microphoto contrivance FMN-3, ocular K10×, apochromatic objective ×90). (A) low basophilic, thin hyphae and their fragments, containing volutin (strain LS-T293); (B) fragment chains of basophilic hyphae, single fragments, spores, and single colonies with high basophilic "young" hyphae (strain BS-21); (C) the difference from strain LS-T293 consists in the presence of a greater amount of the hyphal fragments and volutin (mutant no. 80); (D) the difference from strain BS-21 consists in thicker and more basophilic fragments (mutant no. 49); (E) moderate, basophilic, thin hyphae, containing single volutin granules, fragment chains of basophilic hyphae, single fragments, and spores (hybrid of type I); (F) moderate basophilic hyphae, containing single volutin granules, fragment chains of thick, basophilic hyphae, single fragments and spores (hybrid of type II).*

and morphological characteristics, as well as their cytological features (Fig. 1 to 3). In addition, both strains respond differently to an increase of soluble inorganic phosphorus in the media, and the optimal concentration of corn steep liquor in the media for the growth of strain BS-21 is 0.25 to 0.5%, whereas for strain LS-T293 it is 0.5 to 0.75%.

The data on the comparative study of the prototrophic recombinants of type I and II from crossing 49 × 80 with respect to the above properties are presented in Table 3 and Fig. 1 to 3.

The examination of these data shows that only a few properties of the starting strains, such as the response to streptomycin, and the response to an increase of inorganic soluble phosphorus in the media are inherited by the prototrophs without any change. These properties may be inherited by the prototrophs either from one starting strain, or the other as in inheritance of the reaction to streptomycin. In some cases the inheritance is intermediate, such as the cytological structure of mycelia and the inheritance of the sensitivity to X irradiation, whereas in other cases new properties, such as new cultural and morphological features or the absence of foaming in the fermentation medium are observed. When attempting to induce nonactive variants in prototrophic recombinants by using ultraviolet or X irradiation, an interesting new property of the prototrophic recombinants was shown. A great number of colonies was examined, and very few such variants were detected, although the starting strains LS-T293 and BS-21 produced many such variants after irradiation with ultraviolet or X rays.

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TABLE 3. *Characteristics of Streptomyces rimosus strains LS-T293 and BS-21 as compared with those of hybrids from combination 49 × 80*

Properties studied	Strain LS-T293	Strain BS-21	Hybrids from combination 49 × 80	
			Type I	Type II
Cultural and morphological properties	Substrate mycelium brown. Aerial mycelium grayish	Substrate mycelium brown. Aerial mycelium white	Substrate mycelium almost black. Aerial mycelium white	Substrate mycelium brown. No aerial mycelium
Cytological structure	Partial fragmentation of mycelium in 48 hr. Volutin is formed after 24 hr of growth	Complete fragmentation of mycelium in 48 hr. Volutin is formed at the end of fermentation	Intermediate	Intermediate
Antibiotic activity on usual medium	2,500 μg/ml	1,500 μg/ml	2,650 μg/ml	1,850 μg/ml
Response to increased inorganic soluble phosphorus in the medium	Increased activity	Decreased activity	As in strain LS-T293	As in strain LS-T293
Sensitivity to X irradiation	Sensitive	Resistant	Intermediate	More resistant than strain BS-21
Induced variation (occurrence of nonactive variants)	Frequent	Rare	Very rare	None
Response to streptomycin	Sensitive	Resistant	As in strain LS-T293	As in strain BS-21
Foaming of the fermentation medium	Abundant	Abundant	None	Abundant