POSSIBLE UTILITY OF A FLUORESCENT ANTIBODY TECHNIQUE IN THE SEROLOGICAL IDENTIFICATION OF ANTAGONISTIC *STREPTOMYCES*

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ABSTRACT

ARAI, TADASHI (Chiba University, Chiba, Japan), SHYUKO KURODA, AND MICHIO ITO. Possible utility of a fluorescent antibody technique in the serological identification of antagonistic *Streptomyces*. J. Bacteriol. **83**:20–26. 1962.—The indirect fluorescent antibody technique was extended to the serological investigation of antagonistic *Streptomyces*. Serological relationship was studied among groups of antagonistic *Streptomyces* that are related in their ability to produce antibiotics or in their morphological and cultural characteristics. It was also demonstrated that spores and sporophores are as satisfactorily stained as shake-cultured vegetative mycelium.

Although more than 15 years have passed since active research on newer antibiotics of *Streptomyces* began, and much effort has been made to find adequate criteria for identification and classification of these economically important microorganisms, a conclusive system for their classification has not been achieved.

The criteria used at present mainly involve morphological, cultural, and physiological characteristics. Several attempts have also been made to classify antagonistic *Streptomyces* by means of serological interrelationships. Hata et al. (1953) and Yokoyama and Hata (1953) demonstrated that several strains of antagonistic *Streptomyces* can be identified by agglutination, precipitation, or hemagglutination tests. Okami (1956) reported on the antigenic structure of three antibiotic-producing members of the *S. lavendulae* group. Solovieva, Elpiner, and Fadeeva (1956) also applied serological reactions to the identification of antagonistic *Streptomyces* and noted the superiority of ultrasonically disintegrated mycelium as an antigen. Mayama (1957, 1959), Harada and Kubo (1959), and Tanaka et al. (1959) described serological relationships among species of *Streptomyces*, with special reference to antibiotic production. Slack and his associates (1960, 1961) have recently studied actinomycetes by the fluorescent antibody technique.

In the present series of experiments, the authors attempted to develop a simple and rapid method for the serological classification and identification of antagonistic *Streptomyces* by this technique.

MATERIALS AND METHODS

The test strains of *Streptomyces* listed in Table 1 were maintained on potato agar slants. For the preparation of antigen to immunize rabbits, cultures were grown in a medium containing glucose, 0.5%; starch, 0.5%; meat extract, 0.5%; peptone, 1.0%; and NaCl, 0.3%. After shaking the culture at 27 C for 72 hr, maximal growth was obtained. The fungal bodies were harvested, washed repeatedly with distilled water, and lyophilized. These dried materials were ground in a mortar and suspended in saline. The inoculum size varied from 30 to 60 mg dry weight, depending upon toxicity. Each antigen was injected intravenously into two rabbits every 2 days for a total of 8 to 10 injections. The rabbits were then allowed to rest for 7 days, and were then bled by cardiac puncture.

The indirect method of staining fluorescent antibody was used. Preparation of goat antibody against rabbit globulin and fluorescent labeling were performed as described previously (Ito and Nishioka, 1959; Ito, 1960), the only difference being that labeled antibody was absorbed with a mixture of several dried mycelia of test *Streptomyces* to eliminate nonspecific staining. Smears of shake-cultured and washed mycelia or sporophores from solid media were made on glass slides. The slides were air-dried and then fixed for 20 min in methanol. Specific antiserum of rabbit origin was placed on the dried smear and allowed to stand for 40 min; it was washed off

with buffered saline, and the preparation was again air-dried. The labeled antiglobulin (1:4) was then overlayed. The slides were rinsed after 1 hr. The light source was a high-pressure mercury lamp (Osram HBO 74, Carl Zeiss, Germany), used in combination with a BG 12 filter. A barrier filter (Y_3, Iwaki Co., Tokyo) was attached to the eyepiece.

TABLE 1. *List of test organisms*

Test organism*	Antibiotic produced
S. albus IFM 1044	—
S. lipmanii ATCC 3331	—
S. lavendulae IMRU 3440-8	Streptothricin
S. virginiae IMRU 3651	Actithiazic acid
S. roseochromogenes H-277	Roseothricin
S. fradiae IFM 1039	Neomycin
S. aureofaciens 741	Chlortetracycline
S. toyocaensis	Toyocamycin
Streptomyces sp. SO-927	Toyocamycin
Streptomyces sp. SO-948	Toyocamycin
S. griseus 8049	Streptomycin
S. griseus red variant SN-14	Streptomycin
S. netropsis NRRL B-2268	Netropsin
S. purpeochromogenes 108	Violacetin
S. vinaceus IFM 1017	Viomycin
S. olivoreticuli 100	Viomycin
S. phaeochromogenes ATCC 3338	—

* ATCC = American Type Culture Collection; IFM = Institute of Food Microbiology, Chiba University; IMRU = Institute of Microbiology, Rutgers University; NRRL = Northern Regional Research Laboratory; other strain designations are those of the initial investigators.

RESULTS

Unstained *Streptomyces* cells gave only negligible autofluorescence, which, in the case of fungi, sometimes interferes with that of stained preparations; a perfectly negative control composed of nonfluorescing organisms was obtainable. As a further control on the serological specificity of the staining reactions, homologous absorption tests were carried out. It was found that a 1:4 dilution of conjugated antisera, when absorbed with homologous antigens, no longer stained smears of these or any other species of *Streptomyces*. The absorption of rabbit antiserum with *Nocardia leishmanii*, to eliminate minor degrees of nonspecific reaction among actinomycetes, was unsuccessful, possibly due to differences between the antigenic structures of these two organisms even though they are closely related so far as morphology and cultural characteristics are concerned. Figures 1 to 3 show vegetative mycelia of *S. fradiae* and *S. lipmanii*, which were positively stained with a 1:4 dilution of conjugated and absorbed antisera. Higher magnification clearly revealed the cell-wall structure of the mycelium. In this series of experiments, as well as in the experiments with aerial mycelium or sporophores,

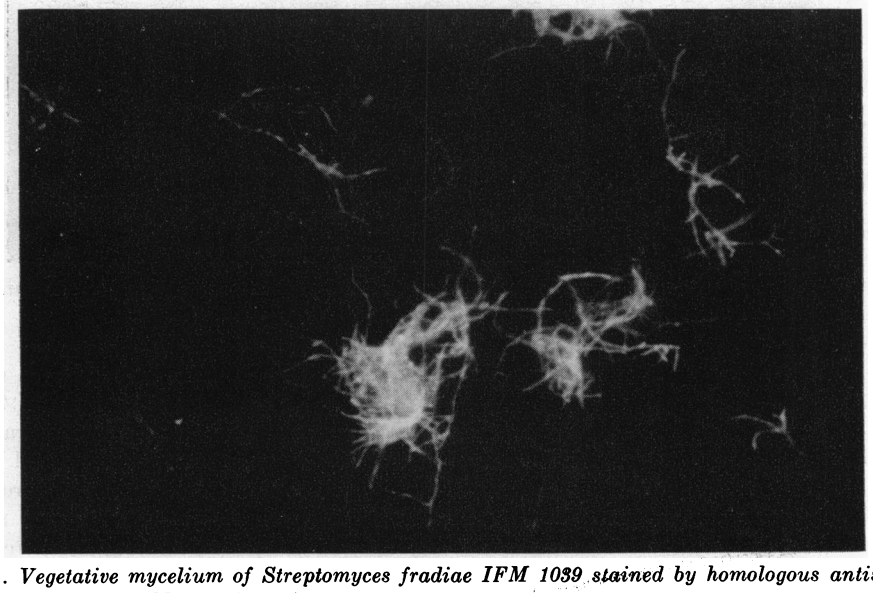

FIG. 1. *Vegetative mycelium of Streptomyces fradiae IFM 1039 stained by homologous antiserum absorbed with S. albus IFM 1044* (×*150*).

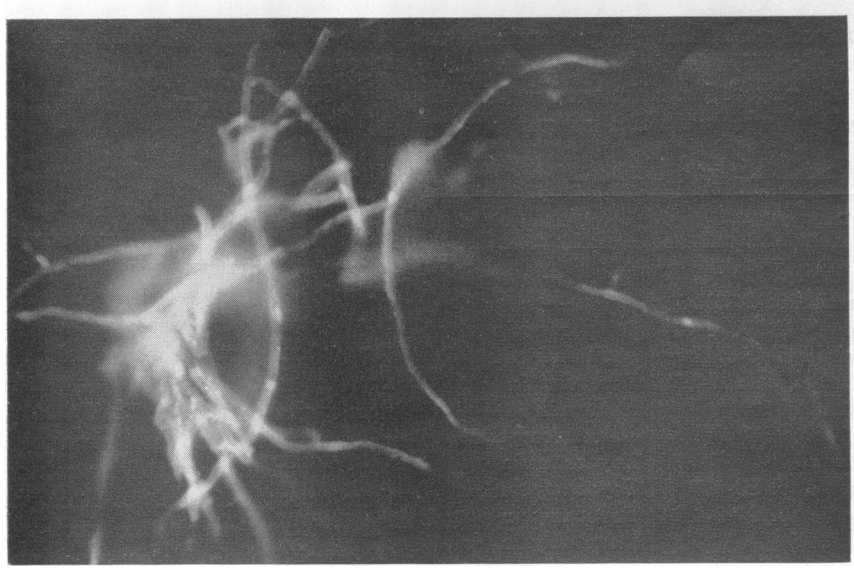

FIG. 2. *Vegetative mycelium of Streptomyces fradiae IFM 1039 stained by homologous antiserum absorbed with S. albus IFM 1044* (×600).

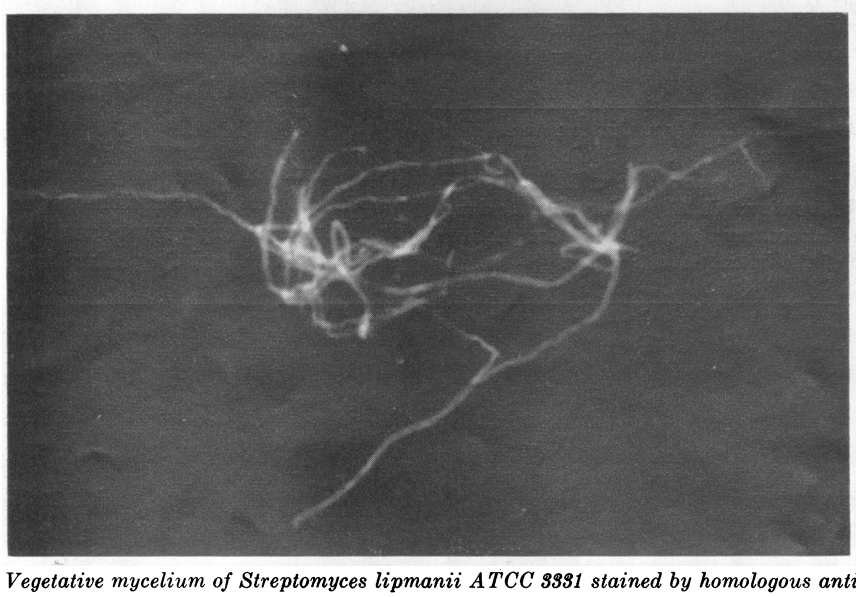

FIG. 3. *Vegetative mycelium of Streptomyces lipmanii ATCC 3331 stained by homologous antiserum absorbed with S. albus IFM 1044* (×600).

the absorption of antisera to eliminate nonspecific reactions was done with organisms which were distinct from the homologous cultures in their morphology, pigmentation, and antibiotic production.

Titration of homologous rabbit antisera by the fluorescent antibody technique was then carried out with the mycelia of *S. griseus* 8049 and *S. fradiae* IFM 1039 (Tables 2 and 3). Unabsorbed *S. griseus* antiserum with a titer of 1:256, when absorbed with *S. phaeochromogenes*, was reduced in titer to 1:64, and it was presumed that both strains share common antigens. Cross-reactions of *S. griseus* with *S. fradiae* antiserum and of *S. fradiae* with *S. griseus* antiserum were removed by absorption of *S. fradiae* antiserum with *S. albus*

TABLE 2. *Titration of immune serum with S. griseus vegetative mycelium as antigen*

Antigen	Immune serum	Antigens used for absorption	Serum dilution								
			1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
S. griseus 8049	*S. griseus* 8049	None	3+*	3+	3+	3+	2+	2+	1+	1+	–
	S. griseus 8049	*S. phaeochromogenes* 3338	3+	3+	3+	2+	1+	1+	–	–	–
	S. aureofaciens 741	None	1+	1+	1+	–	–	–	–	–	–
	S. aureofaciens 741	*S. albus* 1044	–	–	–	–	–	–	–	–	–
	S. fradiae 1039	None	1+	1+	1+	1+	1+	–	–	–	–
	S. fradiae 1039	*S. albus* 1044	1+	–	–	–	–	–	–	–	–

* The three-plus sign indicates the bright greenish-yellow staining reaction, which appears under high power to involve part of the cell-wall structure. Two-plus denotes staining reaction in which cells are less bright, but sharply outlined with greenish-yellow fluorescence. One-plus means that cells are not sharply outlined, but a slight greenish-yellow fluorescence is discernible. Minus means without fluorescence.

TABLE 3. *Titration of immune serum with S. fradiae vegetative mycelium as antigen*

Antigen	Immune serum	Antigens used for absorption	Serum dilution								
			1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
S. fradiae 1039	*S. fradiae* 1039	None	3+*	3+	3+	3+	3+	2+	1+	1+	–
	S. fradiae 1039	*S. albus* 1044	3+	3+	3+	3+	3+	2+	1+	1+	–
	S. griseus 8049	None	3+	2+	1+	±	–	–	–	–	–
	S. griseus 8049	*S. phaeochromogenes* 3338	–	–	–	–	–	–	–	–	–
	S. toyocaensis	None	1+	–	–	–	–	–	–	–	–
	S. toyocaensis	*S. albus* 1044	1+	–	–	–	–	–	–	–	–

* See Table 2 for explanation of symbols.

and by absorption of *S. griseus* antiserum with *S. phaeochromogenes*. Neither *S. griseus* nor *S. fradiae* gave notable titers with heterologous *S. aureofaciens* and *S. toyocaensis* antisera, respectively.

Reciprocal absorption tests were carried out with the mycelia of three groups of *Streptomyces* (Table 4). *S. purpeochromogenes* 108 and *S. netropsis* B-2268 were quite distinct in their cultural characteristics, but the antibiotics obtained from their culture broths were closely related (Aiso et al., 1955). The same was true with *S. olivoreticuli* 100 and *S. vinaceus* IFM 1017, whose major antibiotic was viomycin (Arai, Nakada, and Suzuki, 1957). By immunofluorescence, no reciprocal relationship was noticed between *S. netropsis* and *S. purpeochromogenes*, while a unilateral relationship existed between *S. vinaceus* and *S. olivoreticuli*. A reciprocal relationship was noted between *S. griseus* 8049 and the *S. griseus* red variant.

To determine whether the present procedure is applicable to the serological identification of *Streptomyces* with spores or sporophores, mature sporophores taken from 10-day-old slant cultures of *S. virginiae*, *S. toyocaensis*, and two fresh isolates of *Streptomyces* (SO-927 and 948) were chosen as antigens. Dilutions (1:4) of several selected homologous and heterologous antisera, which were absorbed with *S. albus* or *S. phaeochromogenes*, were used for the staining. Positive staining of *S. virginiae* with *S. lavendulae* antiserum was observed, in addition to staining with each respective homologous antiserum (Table 5). Two fresh isolates were stained with *S. toyocaensis* antiserum. Fluorescent sporophores with homologous antiserum are shown in Fig. 4 and 5.

TABLE 4. *Serological relationships among three selected pairs of antibiotic producers*

Antigen	Antiserum titers (reciprocal)			
	S. netropsis 2268		*S. purpeochromogenes* 108	
	Unabsorbed	Absorbed with *S. purpeochromogenes* 108	Unabsorbed	Absorbed with *S. netropsis* 2268
S. netropsis 2268	256*	256	(8)†	0
S. purpeochromogenes 108	(2)	0	512	256
	S. griseus 8049		*S. griseus* red variant	
	Unabsorbed	Absorbed with *S. griseus* red var.	Unabsorbed	Absorbed with *S. griseus* 8049
S. griseus 8049	256	64	16	0
S. griseus red variant	32	0	512	32
	S. vinaceus 1017		*S. olivoreticuli* 100	
	Unabsorbed	Absorbed with *S. olivoreticuli* 100	Unabsorbed	Absorbed with *S. vinaceus* 212
S. vinaceus 1017	256	128	128	0
S. olivoreticuli 100	(8)	0	1024	1024

* Maximum dilution of antiserum which gave positive reaction.

† Parentheses indicate a possibly nonspecific reaction.

TABLE 5. *Serological identification of antagonistic Streptomyces with sporophores as antigen*

Antigen	Immune serum*			
	S. virginiae 3651	*S. lavendulae* 3440-8	*S. fradiae* 1039	*Streptomyces* SO-927
S. virginiae 3651	3+	3+	−	−
	S. toyocaensis	*Streptomyces* SO-927	*Streptomyces* SO-948	*S. roseochromogenes* H-277
S. toyocaensis	3+†	3+	3+	−
Streptomyces SO-927	3+	3+	3+	−
Streptomyces SO-948	3+	2+	3+	−

* *S. virginiae*, *S. lavendulae*, *S. toyocaensis*, and *Streptomyces* SO-927 immune sera were absorbed with *S. phaeochromogenes*, while *S. fradiae*, *S. roseochromogenes*, and *Streptomyces* SO-948 immune sera were absorbed with *S. albus*, to eliminate nonspecific reactions.

† See Table 2 for explanation of symbols.

DISCUSSION

It is well known that, in agglutination studies of *Actinomycetales*, difficulties are often encountered in the preparation of adequate antigens because of the formation of filamentous and abundantly branched hyphae. Some of the earlier investigators (Ludwig and Hutchinson, 1949; Hata et al., 1953) employed sonic-treated antigens to solve this problem. In this case, however, agglutination and precipitation might have occurred in the same tube. Moreover, strong spontaneous agglutination within a short period of time was sometimes experienced by the present authors with such strains as *S. griseus*, *S. vinaceus*, *S. rimosus*, and *S. olivoreticuli*.

The experimental results summarized here demonstrate that the fluorescein-labeled antibody technique can be successfully applied to the serological investigation of these organisms, thus eliminating the above difficulties. Washed mycelium from the shake-culture in liquid medium, as well as spores and sporophores from the growth on solid agar, were found equally satisfactory as antigens, although the fluorescence with spores alone seemed somewhat weak. It was observed by high-power magnification that the antigen-antibody complex is principally formed on the cell wall.

Although conclusive results were not obtained by reciprocal absorption tests with a limited number of strains, the above findings suggest that this simple technique, which involves only a slide test with washed mycelia or with sporophores, may be useful for the screening of antagonistic *Streptomyces*. If taken together with primary taxonomic observations and the antibacterial spectra on agar plates, the organism in question can be rapidly related with already known antibiotic sources.

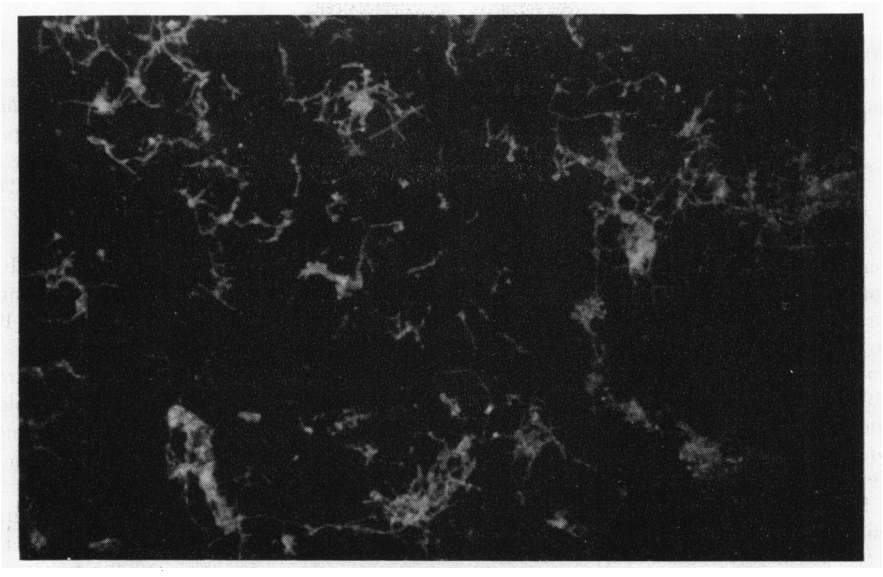

FIG. 4. *Sporophores of Streptomyces toyocaensis stained by homologous unabsorbed antiserum* (×*150*).

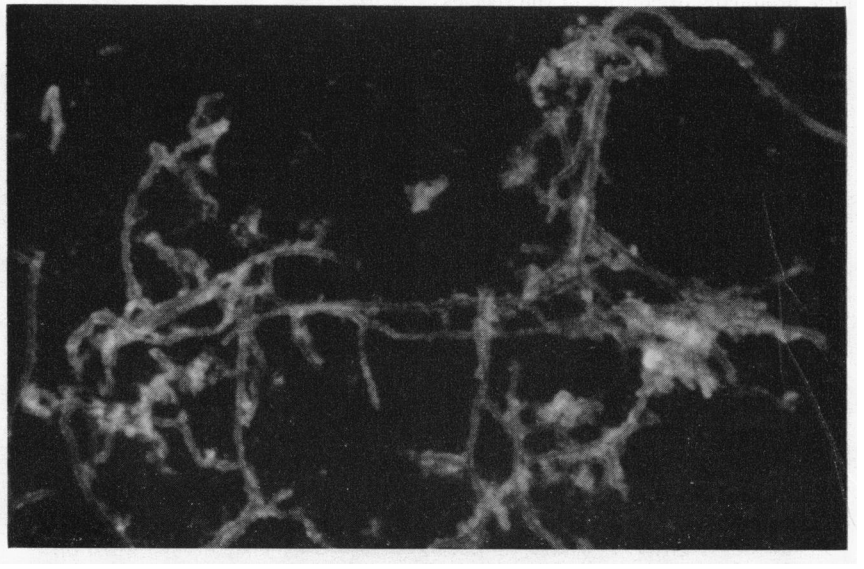

FIG. 5. *Sporophores of Streptomyces toyocaensis stained by homologous unabsorbed antiserum* (×*600*).

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