SEROLOGY OF AEROBIC AQUATIC ACTINOMYCETES

II. Effects of Growth Medium and Culture Age on Antigen Levels

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ABSTRACT

GUTHRIE, R. K. (North Texas State University, Denton), A. W. ROACH, AND J. K. FERGUSON. Serology of aerobic aquatic actinomycetes. II. Effects of growth medium and culture age on antigen levels. J. Bacteriol. **86**:173–178. 1963.—The levels of multiple antigenic components of individual isolates of aerobic actinomycetes were affected by the medium used for culture. The nutritive requirements, and the rate of growth of specific strains on a particular medium, appear to determine the number and titer of antigen components present. The antigenic components present in largest amount reached a peak titer in these organisms in 2 weeks of growth, regardless of medium. At that time, the antigenic components were present primarily in the mycelium. After 2 weeks of growth, additional components appeared in low titer, and those components in largest amount began to appear in the medium. With the appearance of antigenic components in the medium, titers of these antigens decreased in the mycelium. Standardization of conditions of growth medium and culture age served to increase the accuracy and specificity of serological grouping on the basis of reactions observed.

Recently the use of fluorescent-antibody techniques has been reported to make possible the serological grouping of actinomycetes by reciprocal adsorption procedures. Slack, Winger, and Moore (1961) reported the establishment of four serological groups by this procedure in working with *Actinomyces*, *Corynebacterium*, and anaerobic diphtheroid species. By reciprocal adsorption, the number of antigens or haptenes was calculated within these groups. These workers reported that there was no correlation shown between habitat, species designation, and serological grouping, and that no fluorescence was observed when *Streptomyces* and *Nocardia* species were tested with these group antisera.

Arai, Kuroda, and Ito (1962) reported the use of fluorescent antibody in the grouping of several *Streptomyces* species. These workers reported that conclusive results were not obtained by use of this technique with these organisms. Fluorescence in these tests was reported as occurring at the cell wall. Titers were reported for antisera, and the degree of fluorescence was graded visually on a 3+, 2+, 1+ scale. Common antigens were presumed when reciprocal adsorption reduced the degree of fluorescence and the titer.

In both of the reports mentioned above, the presence of multiple antigens or haptenes is assumed or calculated by reciprocal adsorption techniques. The use of fluorescent-antibody procedures does not allow, however, for the direct demonstration or titration of such multiple antigenic components. In the first report in this series (Guthrie, Roach, and Ferguson, 1962), a successful immunization procedure was reported for the production of antiserum to aerobic actinomycetes in rabbits. In that report, the advantages of the use of agar diffusion precipitin tests for titration of multiple antigen or haptene systems were discussed. In studies using these methods for grouping aerobic aquatic actinomycete isolates, antisera were prepared for six culturally different strains. Variable reactions were frequently encountered in the use of individual isolates when tested for cross reactivity using these antisera. An isolate showing two antigen-antibody reactions with antiserum A on one test, for example, would show only one such reaction on a subsequent test. Variations in cross reactivity usually were seen in the precipitin bands of lowest titer. In a few instances, additional precipitin bands were observed when tests were repeated.

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This report deals with the determination of factors which are involved in producing variations observed in these reactions.

MATERIALS AND METHODS

In this study, five actinomycete strains isolated from marine environments were used. In addition, a *S. griseus* strain, NRRL B-150, was included for study of heterologous reactions. For the age studies, all cultures were grown in 200-ml bottles containing Ion Agar (Consolidated Laboratories, Inc., Chicago Heights, Ill.) slants and 30 ml of Trypticase Soy Broth. In this case, each organism was inoculated into seven such bottles and incubated at room temperature. One bottle of each culture was harvested at intervals of 1, 2, 4, 8, 12, 16, and 20 weeks after inoculation. Each test of the effect of age on these cultures was repeated at least once to reduce the chance effects of other factors on these results. Such repeated tests were run at different times.

Cultures for use in the medium study were grown in the same way. Media used, in addition to Trypticase Soy Broth, were Emerson Broth, the medium reported by Arai et al. (1962), and a medium (designated M1B2) developed by Silvey (1963). The latter medium consists of 10 g of sodium citrate, 10 g of glucose, 2 g of potassium nitrate, 2 g of sodium nitrate, 0.1 g of calcium chloride, 0.05 g of magnesium sulfate, and 2 g of dipotassium phosphate in 1 liter of distilled water. This solution was sterilized by filtration through a 0.4-mμ Millipore filter.

Antisera used in this work were prepared in rabbits by the short-schedule immunization previously reported (Guthrie et al., 1962). For all tests, pooled sera from at least two immunized rabbits were used. Agar diffusion precipitation tests were performed as in that report, with the modification that 0.45% Ion Agar was used in preparing plates to permit more rapid diffusion of the reagents.

To prepare actinomycete cultures for testing, the mycelial mat was separated from the culture broth by filtration through Whatman no. 3 filter paper. The broth was frozen for storage until testing. The mycelial mat was washed (three times) with distilled water on the filter and treated by ultrasonic oscillation until uniformly disintegrated. This preparation was bottled and frozen until tested. All preparations were tested within 1 week after cultures were harvested.

RESULTS AND DISCUSSION

In preliminary tests, Trypticase Soy Broth was chosen for the age studies because of the uniformity of growth of all isolates in this medium. Antisera were prepared as early as the culture appeared to have reached maximal growth. The cultures were then tested at the time intervals shown in the results of homologous reactions in Table 1. In the three isolates shown, the maximal titers of antigenic components were reached at 2 weeks of culture, after which time the titers decreased in the mycelium. Titers of antigenic components in the medium began to appear by the second week and increased to a later maximum. There was an indication in the case of strain H44 that a second cycle of antigen production was present at 20 weeks.

That such a second cycle of antigen production occurs in late growth with this organism was also seen in the heterologous reactions (Table 2). In these results, it is seen that, where an isolate consistently cross-reacts with an antiserum, the maximal titer of reaction was reached by the second week in the mycelium. In several instances, cross reactions appeared later than the second week, and in these cases the reaction was seen almost exclusively in the medium rather than in the mycelium. Extended storage of both the disrupted mycelial portion and the broth in a frozen state indicated that the antigenic components were relatively stable compounds. In such storage over a period of several months, some additional cross reactions have been observed, indicating that perhaps some components are complexes which may be disrupted on long storage to form two reactive components.

In observing the gradual decrease in titer of antigen components in the mycelium, with a generally increasing titer in the medium, two explanations appear possible. The first of these would be autolysis of the culture with the release of soluble components. It is thought that this factor does play a role in this shift, but it cannot account for the entire change because of the great variability of autolysis in different isolates.

It is also possible that the antigenic components were slowly released from the intact mycelium to the medium, and that this process was accentuated by any amount of autolysis that occurred. If this were the case, then it appeared that the antigenic components were simply temporary materials residing in the soluble

TABLE 1. *Effect of age on homologous reactions (titers of antigens)**

Isolate no.	Antiserum	Age of culture (weeks)													
		1		2		4		8		12		16		20	
		M	B	M	B	M	B	M	B	M	B	M	B	M	B
H44	H44	1:4	U	1:64	1:4	1:4	1:4	U	1:4	—	1:4	—	U	1.8	1:4
		1:4	—	1:8	U	—	—	—	U	—	—	—	U	—	1:4
		U	—	U	—	—	—	—	—	—	—	—	—	—	—
H55	H55	1:4	—	1:8	1:8	1:4	U	—	1:4	—	1:8	—	1:8	—	1:4
		U	—	U	U	—	—	—	U	—	1:8	—	U	—	—
		—	—	U	—	—	—	—	—	—	1:4	—	U	—	—
H7	H7	1:32	—	1:64	U	1:32	U	—	1:4	—	1:32	—	1:16	—	1:4
		—	—	1:8	—	—	—	—	1:4	—	1:8	—	1:8	—	1:4
		—	—	—	—	—	U	—	U	—	—	—	U	—	—

* Symbols: M = mycelial mat disintegrated by sonic oscillation; B = culture broth; U = undiluted disintegrated mycelial mat; — = negative reaction. Horizontal lines represent multiple antigens as indicated by precipitin bands in each analysis.

TABLE 2. *Effect of age on heterologous reactions (highest titer antigens)**

Isolate no.	Antiserum	Age of culture (weeks)													
		1		2		4		8		12		16		20	
		M	B	M	B	M	B	M	B	M	B	M	B	M	B
H55	H44	—	—	—	—	—	—	—	—	—	1:4	—	—	—	U
H7		1:16	—	1:8	—	1:32	U	—	1:4	—	1:4	—	1:4	—	1:4
H10		1:16	U	1:8	—	1:8	1:4	1:4	U	—	U	—	1:4	—	U
H20		1:8	—	1:4	U	U	U	U	U	—	1:4	—	U	—	U
S. griseus		1:8	—	1:8	U	—	1:4	—	U	—	U	—	U	—	—
H44	H55	1:8	—	1:32	1:4	1:32	1:16	1:8	1:16	U	1:16	—	1:8	1:32	1:8
H7		U	—	—	—	—	—	—	—	—	—	—	—	—	1:8
H10		—	—	—	—	—	—	—	—	—	U	—	—	—	1:4
H20		U	—	1:4	—	U	U	U	U	U	1:4	—	—	—	—
S. griseus		—	—	1:64	1:4	—	1:4	1:4	1:8	—	U	—	1:4	—	U
H55	H7	—	—	—	—	—	—	—	—	—	1:4	—	—	—	—
H44		1:4	—	1:4	—	1:4	U	1:4	U	—	U	—	1:4	U	1:4
H10		1:64	U	1:16	U	1:16	1:4	1:16	U	—	U	—	1:8	—	1:16
H20		1:16	1:4	1:16	1:4	1:4	1:4	U	U	U	1:4	U	1:16	U	1:4
S. griseus		1:32	U	1:32	U	—	U	—	U	—	U	—	U	—	—
H44	H17	U	—	1:8	1:4	U	1:4	U	U	—	—	—	1:4	—	—
H55		—	—	—	—	—	—	—	—	—	1:4	—	—	—	—
H7		—	—	—	—	—	1:16	—	1:4	—	U	—	—	—	—
H10		U	—	1:4	1:8	—	—	—	—	—	—	—	—	—	—
H20		—	—	—	—	U	1:4	—	—	—	—	—	1:4	—	—
S. griseus		—	—	—	—	—	—	—	—	—	—	—	—	—	—

* Symbols: M = mycelial mat disintegrated by sonic oscillation; B = culture broth; U = undiluted disintegrated mycelial mat; — = negative reaction.

portion of the mycelium, and not actually a part of the structure of the organism. To test for the amount of attached antigenic components, mycelium was subjected to sonic oscillation until it was disintegrated. Microscopy of this material revealed only a rare mycelial element intact. This sonically treated material was repeatedly centrifuged and washed with saline. The wash supernatants were tested for the presence of antigenic components. The supernatant fluid from the second wash showed only one antigenic component of four still present in the undiluted saline. Later washings were negative. Specific antiserum was then treated with a portion of the mycelial sediment which had been washed three times. After adsorption for 2 hr at refrigerator temperature, the sediment was removed by centrifugation (2,000 rev/min for 20 min), and the supernatant serum was tested against the complete sonically disrupted mycelial mat by dilution of the serum. After adsorption, there was an eightfold reduction in titer of the antiserum for all four antigen components, indicating that the washed mycelial fragments possessed a considerable amount of the antigenic components in some fixed form.

It appeared that the pH of the culture medium did not influence the presence or titer of the antigenic components, since the pH in all cases changed no more than from a pH of 8.4 to 8.8 over the 20-week culture period. The major effect of culture age would appear to be the location of the antigenic components in either the mycelium or the broth, rather than the absolute titer changes which may occur with time.

From the results above, 2-week cultures were selected for testing the effect of growth medium on antigenic component titer. In Table 3, homologous reactions of cultures in the four broths are shown. Isolate H7 did not grow in the M1B2 medium, and H44 showed poor growth in the medium of Arai et al. (1962). Trypticase Soy Broth showed the greatest production of antigenic components, generally at highest titer, whereas M1B2 showed the least release of antigenic components into the medium, with the lowest mycelial component titers.

In the heterologous reactions shown in Table 4, little difference was seen in the number of cross reactions produced by cultures in any one medium, although there was considerable difference in the production of cross reactions in single strains on different media. For example, H7 grown on Trypticase Soy Broth showed a cross reaction only with H44 antiserum, but H7 grown on Emerson Broth reacted with H55, H44, and H17 antisera. Poor growth of H44 in the medium of Arai et al. (1962) and lack of growth of H7, H20, and H10 in M1B2 medium prohibit the use of these media for this work, although in examination of multiplicity of antigen components cross-reacting in several isolates these

TABLE 3. *Effect of medium on homologous reactions (2-week cultures)**

Isolate no.	Antiserum	Trypticase Soy		Emerson		Arai†		M1B2	
		Mat	Broth	Mat	Broth	Mat	Broth	Mat	Broth
H55	H55	1:8	1:8	1:16	1:8	1:4	1:32	1:4	—
		U	U	1:16	1:8	1:4	1:32	1:4	—
		U	—	—	—	—	1:8	—	—
H7	H7	1:64	U	1:8	U	1:64	U	NG	NG
		1:8	—	—	U	1:16	U	NG	NG
H44	H44	1:64	1:4	1:8	1:4	U‡	—‡	1:16	—
		1:8	U	U	—	—‡	—‡	1:4	—
		U	—	—	—	—‡	—‡	—	—

* Symbols: — = negative reaction; U = undiluted disintegrated mycelial mat; NG = no growth. Horizontal lines represent multiple antigens as indicated by precipitin bands in each analysis.

† Medium reported by Arai et al. (1962).

‡ Poor growth of H44 on Arai medium.

TABLE 4. *Effect of medium on heterologous reactions (highest titer antigens at 2 weeks)**

Isolate no.	Antiserum	Trypticase Soy		Emerson		Arai†		M1B2	
		Mat	Broth	Mat	Broth	Mat	Broth	Mat	Broth
H7	H55	—	—	1:4	—	1:16	—	NG	NG
H44		1:32	1:4	1:16	—	U‡	—‡	1:8	—
H20		1:4	—	—	—	1:8	—	NG	NG
H10		—	—	1:16	—	1:8	1:4	NG	NG
S. griseus		1:64	1:4	1:8	1:4	1:8	—	1:4	—
H7	H44	1:8	—	1:4	—	1:32	—	NG	NG
H55		—	—	U	1:4	U	1:8	U	—
H20		1:4	U	1:4	U	1:8	—	NG	NG
H10		1:8	—	1:16	—	1:4	U	NG	NG
S. griseus		1:16	U	1:4	U	1:8	—	U	—
H7	H17	—	—	—	U	—	—	NG	NG
H44		1:8	1:4	1:8	1:4	—‡	—‡	—	—
H55		—	—	—	—	—	1:4	—	—
H20		—	—	—	—	—	—	NG	NG
H10		1:4	1:8	—	—	—	—	NG	NG
S. griseus		—	—	—	—	—	—	—	—
H44	H7	1:4	—	1:16	—	—‡	—‡	U	—
H55		—	—	—	—	—	1:4	—	—
H20		1:16	1:4	1:8	U	1:16	U	NG	NG
H10		1:16	U	1:32	U	1:8	1:4	NG	NG
S. griseus		1:32	U	—	—	—	—	U	—

* Symbols: — = negative reaction; U = undiluted disintegrated mycelial mat; NG = no growth.
† Medium reported by Arai et al. (1962).
‡ Poor growth of H44 on Arai medium.

media appear to provide the greatest reduction in number of cross reactions.

The variation of antigen components with age of culture and on different media makes necessary the standardization of these conditions for serological study of these organisms. In earlier work on this project, a survey of cross reactions with no limitation of culture age, and with use of both Trypticase Soy and Emerson Broth cultures, was run. This survey has been repeated using 2-week cultures in Trypticase Soy Broth for antiserum production and testing. These surveys are compared in Table 5.

In these results, it was seen that the factors of age and culture medium presented a most confusing picture when compared with the controlled results. The greatest variations were shown in reactions with H55 and H38 antisera. In the case of H55, reactions were increased by standardization of these conditions. In the case of H38, reactions were decreased by this standardization.

TABLE 5. *Number of reacting antigen components in standardized cultures vs. random cultures**

Isolate no.	Antiserum								
	H7		H55		H20		H38	
	A	B	A	B	A	B	A	B
H55	—	—	2	5	—	1	2	—
H7	5	2	—	3	—	1	1	2
H20	—	—	—	2	2	3	1	—
H38	—	1	—	2	—	1	3	3
H10	3	2	—	3	—	—	2	—
H24	—	1	—	3	—	—	2	—
H30	—	—	1	3	—	—	2	1
H31	1	2	—	2	—	—	2	—
H48	1	1	—	—	—	—	—	—
H72	1	1	—	—	—	—	2	—

* A = random cultures, not restricted as to medium or age; B = 2-week cultures in Trypticase Soy Broth; — = negative reaction.

The nutritive requirements and the rate of growth are probably responsible for these results. With this demonstrated variability, the absolute necessity for standardization of these conditions for serological study of these organisms is established. From these results, the 2-week age standard appeared to be optimal. Of the four media tested, Trypticase Soy appeared preferable on the basis of all reactions observed. However, a chemically defined medium, containing the least amount of organic material which will support adequate growth of all strains, would seem to be most desirable. Efforts toward the development of such a culture medium are continuing in this laboratory. Work involving the use of identified strains of organisms grown under standard conditions as above for production of antisera, and for use in reciprocal adsorption studies, is underway in this laboratory.

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