STUDIES WITH MICROAEROPHILIC ACTINOMYCETES

II. Serological Groups as Determined by the Reciprocal Agglutinin Adsorption Technique[1, 2]

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Reciprocal agglutinin adsorption procedures have been applied to serological studies with several microorganisms such as: *Shigella* (Ewing, 1953), *Escherichia* (Kauffmann, 1951; Orskov, 1952), *Paracolobactrum* (Eveland and Faber, 1953; Edwards *et al.*, 1948), *Lactobacillus* (Williams *et al.*, 1953; Orland, 1950), *Streptococcus* (Lancefield, 1940*a*, 1940*b*; Watson *et al.*, 1944), and *Cryptococcus* (Evans, 1949 and 1950; Benham, 1935).

The agglutinin adsorption technique has been applied to the actinomycetes by Aoki (1936), Lentze (1938) and Erikson (1940). The latter author also utilized the mirror adsorption technique but no instances of identical mirror absorptions were found.

Cross agglutination between microaerophilic actinomycetes isolated from a variety of sources had been demonstrated by Slack *et al.* (1951), but these reactions did not provide adequate information concerning the antigenic similarities or differences among these actinomycetes. Thus, the reciprocal agglutinin adsorption technique was employed to furnish additional information about the antigenic composition of these microorganisms.

Much of the literature dealing with the reciprocal agglutinin adsorption procedures is sparse in its descriptions of the technique and there was usually little attempt to standardize or follow a uniform procedure. It was necessary to standardize our procedure as much as possible so that the ratio between the antiserum and the adsorbing homologous or heterologous antigen would be uniform. Such a method was described by Krumwiede *et al.* (1925), thus our basic procedures follow those described by this author.

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MATERIALS AND METHODS

Twenty cultures of microaerophilic actinomycetes were used in this study. The laboratory number and source of each culture is given below:

A-1, A-2, A-4, A-5, A-6, A-11, A-13 and A-21 were originally isolated from human cases of actinomycosis.

B-2, B-6, B-7 and B-8 were isolated from cases of bovine actinomycosis.

E-1 and E-3 were isolated from equine actinomycosis.

P-1 was isolated from porcine actinomycosis.

M-1 was from a case of human pyorrhea.

T-12, T-43, T-48 and T-97 were isolated from human nonactinomycotic tonsils.

For more detailed information concerning the source of these cultures see Slack *et al.* (1951).

Each culture was first subcultured in nonantigenic peptone dialysate thioglycolate medium (Slack *et al.*, 1951) and this medium was exclusively used for the cultivation of all organisms. The nonantigenicity of such medium has been further substantiated by the diffusion-precipitin studies of Jennings (1954).

Immunizing antigens were prepared from each of the above cultures using large centrifuge bottles containing 150 ml of peptone dialysate medium plus 20 mg per cent of sodium thioglycolate and inoculating each with 0.1 ml of a concentrated suspension of an actively growing 4-day culture. Following 6 days of incubation at 37 C, the bottles were centrifuged for 30 min at 2000 rpm. The supernatant fluid was decanted

and the actinomycete cells were washed three separate times in fresh 0.85 per cent saline. After the third washing the resuspended cells were transferred to a graduated tube and after centrifuging, 25 ml of 1:10,000 merthiolated normal saline was added for each 0.1 ml of packed cells. This suspension was incubated for 48 to 52 hours at 37 C and then tested for sterility by culturing in thioglycolate medium.

A pair of rabbits whose normal sera reacted negatively to the antigen was then immunized with each culture. During the first 6 days each rabbit received successively 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 ml of the antigen suspension intravenously. After a week's rest they received 2.0 ml of antigen intravenously for each of the next 6 days. After another week's rest a final series of 6 daily 2.0-ml intravenous injections of the antigen were given. Trial bleedings indicated agglutinin titers of 1:1280 to 1:10,240 for the homologous antigen, thus the rabbits were bled out by cardiac puncture a week after the last injection. Antiserum was collected from the clotted blood and merthiolate in the final concentration of 1:10,000 was added. The antiserum from each pair of rabbits was pooled, distributed into vials in 10-ml amounts and stored in the deep freeze. When ready for use, a single vial was removed and kept in the refrigerator until empty.

The final titer for each pooled antiserum was as follows:

A-1, A-21, E-1, E-3, P-1, M-1, T-12, T-48	1:1280
A-4, A-6	1:2560
A-2, A-11, B-6, B-7, B-8, T-43, T-97	1:5120
A-5, A-13, B-2	1:10,240

Antigens used for the agglutinin and agglutinin adsorption tests were prepared and stored in the same manner as described above, except that merthiolate was not added after the third saline washing.

The agglutination test procedure used in the determination of agglutinin titers as well as for testing the antiserum after it had been adsorbed was standardized as follows: 0.5 ml of 1:10 dilution of the antiserum was placed in the first tube and then 2-fold dilutions were made through a final dilution of 1:5120. The antigen was removed from the refrigerator and adjusted to a turbidity of 500 (±25) "Nephelos" units using a Model 7 Coleman Nephelometer. Then 0.5 ml of this antigen suspension was added to each tube giving final antiserum dilutions of 1:20, 1:40, 1:80, etc., to 1:10,240. Serum and saline controls were included. The tubes were incubated at 37 C for 2 hours, placed in the refrigerator overnight, and readings were taken the following morning. Titers were recorded as the highest dilution of antiserum which produced macroscopically visible agglutination of the antigen.

Krumwiede *et al.* (1925) reviewed the agglutinin adsorption techniques in great detail and in their descriptions they include a method for calculating the amount of antiserum to be added to a given volume of cells. The following formulae were used:

$$(\text{Cell mass} - 10\%) \times \text{Antigen dose factor} = \text{Total volume of antiserum plus cells}$$

$$\frac{(\text{Cell mass} - 10\%) \times (\text{Antigen dose factor} - 1)}{\text{Serum dilution factor}} = \text{Amount of undiluted antiserum required}$$

In performing the agglutinin adsorption procedures with the actinomycetes the washed antigen was removed from the refrigerator, transferred to a graduated centrifuge tube and packed by centrifugation. This cell mass volume was then substituted in the above formulae and the amount of antiserum to be added to this particular volume of cells was calculated.

This calculated amount of antiserum was then added to the cells and incubated at 55 C for 2 hours with shaking by hand every 15 minutes, followed by overnight refrigeration. An extended series of experiments demonstrated that this combination of temperature and time with shaking provided the maximum amount of agglutinin adsorption.

The cells were then packed by centrifugation and the once adsorbed antiserum was added to a like volume of fresh cells. This antigen-antiserum suspension was again incubated, shaken and refrigerated. A third adsorption using fresh cells was then carried out. An agglutination procedure was then done using the adsorbed antiserum and the adsorbing antigen in order to determine whether or not the agglutinins had been completely removed. It was found that three such adsorptions were sufficient in every instance to remove all of the homologous agglutinins.

An extended series of experiments was then done to determine the amount of heterologous antigen required to adsorb completely the ag-

TABLE 1

Agglutinin adsorption procedure using A-1 antiserum, the homologous A-1 antigen and the heterologous A-2 antigen

Homologous Adsorption	Heterologous Adsorption
A-1 antiserum + A-1 cells (adsorbing dose)	A-1 antiserum + A-2 cells (1.5–3 times homologous adsorbing dose)
Adsorption repeated 3 times, each time with fresh cells	Adsorption repeated 3 times, each time with fresh cells
Agglutination test using A-1 antigen	Agglutination test using A-1 and A-2 antigens

glutinins from an antiserum. It was finally determined that an amount of heterologous antigen equivalent to 1.5–3 times the adsorbing dose of homologous antigen was necessary to accomplish this adsorption. Otherwise the procedure was the same as described above.

An outline of the adopted cross agglutinin adsorption procedures using A-1 antiserum with A-1 and A-2 antigens is given in table 1.

These procedures were then extended to the reciprocal agglutinin adsorption technique in which, for example, A-1 and A-2 antisera were each divided into two portions and adsorbed respectively by A-1 and A-2 antigens. Then agglutination tests were done on each sample of adsorbed antiserum using these two antigens. This procedure was repeated using A-1 and A-4 antisera with A-1 and A-4 antigens, A-1 and A-5 antisera with A-1 and A-5 antigens, etc.

RESULTS

The results obtained using A-1, A-2 and A-4 antisera and antigens are given in table 2.

Identical results were obtained when using the antigens and antisera of A-1, A-2, A-4, A-11, A-13, A-21, B-2, E-3, P-1, M-1, T-12, T-43, T-48 and T-97.

These findings indicate that these actinomycetes have similar complete and partial antigens. It should be emphasized that these microorganisms include those isolated from cases of human, bovine, equine, and porcine actinomycosis as well as from the mouth and tonsils of humans not suffering from actinomycosis.

In doing the reciprocal adsorption test with A-1, and the other actinomycete antigens and antisera, the first indication of an antigenic difference was encountered using the A-5 antiserum and A-1 antigen. This series of experiments is recorded in table 3.

It should be emphasized that even three additional adsorptions with fresh A-1 cells failed to reduce the A-5 antiserum homologous agglutinin titer below 1:640.

Thus, there was at least one actinomycetes isolated from a human case of actinomycosis which had certain antigenic or haptenic components differing from the A-1 culture.

Table 4 lists the results showing additional actinomycetes which have antigens differing from those present in the A-1 cells.

These results show that there are microaerophilic actinomycetes isolated from cases of human, bovine and equine actinomycosis which are in themselves antigenically similar but at the

TABLE 2

Reciprocal agglutinin adsorption tests using A-1, A-2, A-4, antisera and antigens

Antiserum	Cells Used for Adsorption	Antigens Used to Test Adsorbed Antiserum	Titer
A-1 (titer 1:1,280)	A-1	A-1	0
A-2 (titer 1:5,120)	A-2	A-2	0
A-1	A-2	A-1	0
A-1	A-2	A-2	0
A-2	A-1	A-1	0
A-2	A-1	A-2	0
A-1 (titer 1:1,280)	A-1	A-1	0
A 4 (titer 1:2,560)	A-4	A-4	0
A-1	A-4	A-1	0
A-1	A-4	A-4	0
A-4	A-1	A-1	0
A-4	A-1	A-4	0

TABLE 3

A-5 antiserum titers following 3 successive agglutinin adsorptions with A-1 antigen

	Before Adsorption	After First Adsorption	After Second Adsorption	After Third Adsorption
A-5 titer.....	1:10,240	1:1,280	1:640	1:640
A-1 titer.....	1:2,560	1:640	0	0

TABLE 4

Reciprocal agglutinin adsorption tests showing the cultures having antigens differing from those in A-1

Antiserum	Cells Used for Adsorption	Antigens Used to Test Adsorbed Antiserum	Titer
A-5 (titer 1:10,240)	A-5	A-5	0
A-1 (titer 1:1,280)	A-5	A-1	0
A-1	A-5	A-5	0
A-5	A-1	A-1	0
A-5	A-1	A-5	1:640
A-6 (titer 1:5,120)	A-6	A-6	0
A-1	A-6	A-1	0
A-1	A-6	A-6	0
A-6	A-1	A-1	0
A-6	A-1	A-6	1:320
B-6 (titer 1:5,120)	B-6	B-6	0
A-1	B-6	A-1	0
A-1	B-6	B-6	0
B-6	A-1	A-1	0
B-6	A-1	B-6	1:160
B-7 (titer 1:5,120)	B-7	B-7	0
A-1	B-7	A-1	0
A-1	B-7	B-7	0
B-7	A-1	A-1	0
B-7	A-1	B-7	1:640
B-8 (titer 1:5,120)	B-8	B-8	0
A-1	B-8	A-1	0
A-1	B-8	B-8	0
B-8	A-1	A-1	0
B-8	A-1	B-8	1:160
E-1 (titer 1:1,280)	E-1	E-1	0
A-1	E-1	A-1	0
A-1	E-1	E-1	0
E-1	A-1	A-1	0
E-1	A-1	E-1	1:80

same time differing antigenically from actinomycetes isolated from like sources.

The next step was to determine whether or not all of the organisms which did not adsorb all the agglutinins from A-1 antiserum were in themselves antigenically similar. Thus, reciprocal agglutinin adsorption tests were done using A-5 antiserum and A-5 antigen with antisera and antigens of A-6, B-6, B-7, B-8 and E-1. In each instance complete reciprocal adsorptions were obtained demonstrating that these actinomycetes within themselves are antigenically similar. However, they have either antigens or haptens or both which differ from those present in the first group.

TABLE 5

Serological grouping of twenty actinomycetes

Group A	Source	Group B
A-1, A-2, A-4, A-11, A-13, A-21	Human actinomycosis	A-5, A-6
B-2	Bovine actinomycosis	B-6, B-7, B-8
E-3	Equine actinomycosis	E-1
P-1	Porcine actinomycosis	—
M-1	Human pyorrhea	—
T-12, T-43, T-48, T-97	Human non-actinomycotic tonsils	—

These experiments demonstrate that the twenty cultures of actinomycetes isolated from human, bovine and porcine sources may be divided into two serological groups and the actinomycetes within each group are antigenically similar. These two groups are designated as A and B and include the actinomycetes as shown in table 5.

DISCUSSION

Identification and classification within the genus *Actinomyces* has been controversial for some 70 years and has resulted in the publication of numerous generic and species names. However, during this time there has been little progress in the application of serological procedures to these problems.

The above experiments demonstrate that the 20 cultures of microaerophilic actinomycetes can be divided into two serological groups on the basis of the reciprocal agglutinin adsorption technique. The members of either group include organisms isolated from human, bovine, equine or porcine sources. Thus, habitat did not dictate their antigenic composition. These results would tend to cast doubt upon the validity of using source or habitat as a basis of origin of species names.

It is tempting to say that all of the actinomycetes which will reciprocally absorb the agglutinins from each other's antisera should be considered as having identical antigens. However, it is

possible that subsequent chemical, chromatographic or agar diffusion studies may reveal certain antigenic or haptenic differences among these microorganisms. Thus, following the example of the terminology used in the serological classification of streptococci and Salmonella these groups have been designated as Group A and Group B.

SUMMARY

Twenty cultures of microaerophilic actinomycetes isolated from human and animal sources were used in this study.

Rabbits were immunized with each culture and then reciprocal agglutinin adsorptions were done using these various antigens and antisera.

The results demonstrated that cultures A-1, A-2, A-4, A-11, A-13, A-21, B-2, E-3, P-1, M-1, T-12, T-43, T-48 and T-97 would reciprocally adsorb each other's agglutinins and these actinomycetes were designated as those comprising Group A.

Cultures A-5, A-6, B-6, B-7, B-8 and E-1 would also reciprocally adsorb each other's agglutinins but not those of the above group. These were designated as Group B.

Thus, there are microaerophilic actinomycetes isolated from human, bovine, equine and porcine sources which may be divided into two serological groups. Group A includes fourteen actinomycetes, some of which were isolated from all the above sources. Group B contains six cultures, each isolated from either human, bovine or porcine actinomycosis. From these results it is apparent that habitat does not correlate with antigenic composition.

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