The Serological Identification of Streptomycetes by Agar Gel Diffusion Techniques

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SUMMARY

The agar gel diffusion technique was used to investigate the serological relationships between species and strains of *Streptomyces*. A method is described for the rapid production of antisera which show specific reactions and also a multiplicity of cross-reactions. The technique has been used for the identification and comparison of antibiotic-producing isolates of streptomycetes from soil.

INTRODUCTION

The taxonomy of the genus *Streptomyces* is complex and at present confused. Classifications based on morphology, biochemical characteristics and antibiotic production have been described and recently reviewed by Waksman (1961), but no comprehensive serological method has been accepted for identifying species of Streptomycetes. Previous serological studies of this genus have used conventional precipitation, agglutination or complement-fixation techniques (Aoki, 1935, 1936*a*, *b*; Ludwig & Hutchinson, 1949; Slack, Ludwig, Bird & Canby, 1951; Okami, 1956; Kuroya, Katafiri, Sato & Mayama, 1958) with the result that, although the antisera showed some species specificity, frequent cross-reactions occurred between apparently unrelated species. Somatic antigens common to several strains of Streptomycetes have been demonstrated by haemagglutination (Hata, Ohki, Yokoyama & Koga, 1953; Yokoyama & Hata, 1953), and recently the presence of group and specific antigens in other species was confirmed by Douglas & Garrard (1958) by passive haemagglutination. The difficulties encountered in this earlier work can be attributed mainly to the multiplicity of cross-reactions. An alternative technique, the agar gel double-diffusion technique (Ouchterlony, 1948), has been used to examine complex antigenic systems in bacteria and this paper describes its application to the genus *Streptomyces*.

METHODS

Organisms. Details of the organisms used in this study are given in Table 1, with the appropriate strain number and culture collection initials, i.e. NCTC (National Collection of Type Cultures, Colindale, London, England), NCIB (National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland), NRRL (North Regional Research Laboratories, Peoria, Illinois, U.S.A.), IMRU (Institute for Microbiology, Rutgers University, New Jersey, U.S.A.) or B/FD (Research Department, Boots Pure Drug Co. Ltd, Nottingham, England). All of these organisms had been maintained as freeze-dried cultures for varying periods.

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Preparation of antigens. Cultures were prepared by inoculating spores or vegetative organism into 500 ml. flasks containing 100 ml. sterile PYG broth (% w/v: peptone, Difco, 0.5; yeast extract, Difco, 0.5; glucose, 1; Casamino acids, Difco, 0.1; NaCl, 0.5; pH 7.0–7.2). Good growth of streptomycetes was usually obtained after incubation for 3–4 days on a rotary shaker at 28°; bacteria were harvested after 2 days at 30°. Organisms were collected by centrifugation, washed twice in saline and resuspended in saline containing 0.01 % (w/v) thiomersalate. The concentration of the resulting suspensions, which were used for immunizing rabbits and in the diffusion plates, was standardized to 60 mg. dry weight organism/ml.

Table 1. *Organisms used*

Strain	Source	Strain	Source
<i>Streptomyces albus</i> 3525	NCTC	<i>Streptomyces</i> sp. 2288	NRRL
<i>S. albus</i> B1337	NRRL	<i>Streptomyces</i> sp. A9257	Soil isolate
<i>S. antibioticus</i> 6124	NCTC	<i>Streptomyces</i> sp. B4049	Soil isolate
<i>S. albogriseolus</i> 1305	NRRL	<i>Streptomyces</i> sp. B2886	Soil isolate
<i>S. aureofaciens</i> 2209	NRRL	<i>Streptomyces</i> sp. L1652	Soil isolate
<i>S. coelicolor</i> B1260	NRRL	<i>Bacillus subtilis</i> 8236	NCTC
<i>S. erythreus</i> 903	B/FD	<i>Candida albicans</i> F1/52	NCTC
<i>S. erythreus</i> 8594	NCIB	<i>Corynebacterium pyogenes</i>	B/FD
<i>S. fradiae</i> 474	B/FD	<i>Dermatophilus dermatonomus</i>	B/FD
<i>S. griseus</i> B/FD 196	IMRU	<i>Mycobacterium</i> sp. 607	B/FD
<i>S. lavendulae</i> 3440-8	IMRU		
<i>S. lavendulae</i> 3516	IMRU		
<i>S. lavendulae</i> 3531	IMRU		
<i>S. lavendulae</i> 3542	IMRU		
<i>S. lavendulae</i> 9000	NCIB		

PYG broth was found to be a most useful medium for this work; in all cases it gave diffuse growth in shake-flask culture without the appearance of the pellets which so often occur in other media. The medium used did not appear to affect the antigenicity of the *Streptomyces* species significantly. Antiserum to *Streptomyces* sp. 2288 grown in PYG broth produced the same number of precipitation lines when tested against this strain grown in PYG broth, PG broth (% w/v: peptone, Difco, 1; glucose, 1) or NZA broth (% w/v: NZ amine A, Sheffield Farms, 0.1; peptone, Difco, 0.5; yeast extract, Difco, 0.5; glucose, 1; NaCl, 0.5). However, although the qualitative response was similar, mycelia from PYG or NZA media reacted with lower antiserum concentrations than mycelia grown in PG medium.

Early immunization experiments were made with the above suspensions or reconstituted freeze-dried ones. However, some difficulty was experienced in injecting this material into rabbits, particularly by the intravenous route, and later material was homogenized before being used for injection by disrupting the organisms, without added abrasive, in the Hughes press (Hughes, 1951) at –30°. This may also have liberated additional antibody-determining groups. Evidence to support this was obtained when antiserum prepared against disrupted *Streptomyces antibioticus* 6124 was placed in the centre reservoir of a diffusion plate and alternate outer reservoirs filled with suspensions of normal or disrupted mycelium at equivalent concentrations. In addition to the six precipitation lines adjacent to the normal mycelium, two additional lines were observed between the disrupted mycelium and serum. However, in a similar experiment with *S. lavendulae* 3440-8, additional lines were not observed.

Immunization procedure. Rabbits (2.5–3 kg.) of either sex were fed on Diet 18 pellets and hay *ad libitum*. Intravenous injections of suspensions of homogenized organism were made into the marginal ear vein. When antigen was given intramuscularly it was injected into the flexor muscles of two rabbits twice, at an interval of 1 week. One ml. of a 50 % emulsion of Freund's adjuvant (Difco) containing the equivalent of 30 mg. dry wt. disrupted mycelium/ml. was used. Blood samples were taken from the marginal ear vein at intervals. When satisfactory antibody titres had been attained the rabbits were fasted overnight, anaesthetized with urethane (1.6 g./kg.) followed by chloroform and the animal exsanguinated by heart puncture. The blood was allowed to coagulate, refrigerated overnight and the serum separated by centrifugation. Sera were stored in a deep freeze at -10° as some loss in titre was observed after freeze-drying.

Serological methods. A slight modification of the agar double-diffusion technique of Ouchterlony (1948) was used. The tests were made in 9 cm. Petri dishes containing 20 ml. agar medium. We found that, of several tested, the basal medium described by Crowle (1958) gave the clearest results. The medium contained 1 % 'ion agar' (Oxoid) dissolved in 0.2M sodium barbital + hydrochloric acid buffer (pH 7.4) containing 0.01 % (w/v) thiomersalate. Circular reservoirs were made with a 6 mm. cork borer and sera pipetted into two central reservoirs. The mycelial suspensions were placed in six peripheral reservoirs (Fig. 2). The Petri dishes were incubated in a humid chamber at 26° and examined daily for the appearance of precipitation lines. When fully developed (after 2–4 days) the lines were observed and counted by two independent observers.

Antibody titres were determined by the agar diffusion method which Thorne & Belton (1957) used for antigen titration.

RESULTS

The sera of normal rabbits did not react in agar with mycelial or whole culture preparations of any of the *Streptomyces* species used. A low antibody titre was sometimes obtained by repeated injections of shake-flask culture filtrate concentrated by freeze-drying. High antibody titres (about 1/128) were consistently obtained with mycelial preparations.

Two immunization procedures were compared for maximum antibody production. *Streptomyces lavendulae* 3440-8 disrupted mycelium was used in a comparison with two groups of three rabbits. One group was given a total of equiv. 60 mg. dry wt. antigen with adjuvant in two intramuscular injections with an interval of 1 week. Three weeks later equiv. 12 mg. dry wt. disrupted mycelium was injected intravenously. Rabbits in the other group received the same amount of antigen (equiv. 72 mg. dry wt.) intravenously in eight ascending doses over 4 weeks. Commencing 1 week later samples of serum from all the rabbits were obtained at intervals and the antibody titre determined. Although there was some variation between rabbits of the same group it could be concluded that the maximum titres occurred 6–7 weeks after beginning immunization. The method involving the use of adjuvant gave slightly higher titres and, since with it only three injections had to be made, this procedure was adopted in subsequent work. Serum from rabbits injected with Freund's adjuvant without added *Streptomyces* mycelium gave no precipitation lines when tested against a variety of *Streptomyces* strains.

Specificity of sera

Sera were prepared against seven established *Streptomyces* species and five strains of *Streptomyces lavendulae*. Generic specificity was investigated by testing these antisera against disrupted organisms of the following: *Bacillus subtilis*, *Candida albicans*, *Corynebacterium pyogenes*, *Dermatophilus dermatonomus*, *Mycobacterium* sp. 607. No precipitation lines were seen except one indistinct line against the

Fig. 1. Diagrammatic representation of antisera specificity shown by shading the squares according to the number of precipitation lines observed. Strains of *Streptomyces* represented as follows: (1) *S. albus* 3525, (2) *S. antibioticus* 6124, (3) *S. coelicolor* B1260, (4) *S. erythreus* 903, (5) *S. fradiae* 474, (6) *S. aureofaciens* 2209, (7) *S. albogriseolus* 1305, (8) *S. lavendulae* 3542, (9) *S. lavendulae* 9000, (10) *S. lavendulae* 3440-8, (11) *S. lavendulae* 3531, (12) *S. lavendulae* 3516.

Mycobacterium strain. However, it was found that this was not related to the *Streptomyces* antibodies as sera from apparently normal unimmunized rabbits occasionally produced a similar line.

Each of the twelve antisera was tested at least twice against the twelve *Streptomyces* antigen preparations and the number and appearance of the precipitation lines recorded. The results are given in Fig. 1. The response of homologous pairs

was very marked and, in each case, at least six precipitation lines were observed. Also, the presence of many common antigens was indicated by the large number of cross-reactions. In many cases the cross-reacting lines were weak in comparison with the strong precipitation lines exhibited between homologous antigens and sera.

However, there was a very marked cross-reaction between *Streptomyces albus*, *S. erythreus* and their respective antisera. The diffusion plate showed a very strong pattern of identity, with many common precipitation lines (Fig. 2(a)); a detailed study of the two organisms was therefore undertaken. Their morphology, growth, pigment production and physiological reactions were very similar and quite different from the published descriptions of *S. erythreus* (Waksman & Curtis, 1916).

Fig. 2. Agar diffusion plates (A) showing precipitation lines between antiserum to *Streptomyces erythreus* 903 (4) and (a) *S. albus* 3525, (b) *S. antibioticus* 6124, (c) *S. coelicolor* B1260, (d) *S. erythreus* 903; and (B) showing precipitation lines between antiserum to *S. lavendulae* 9000 (9) and (e) *S. fradiae* 474, (f) *S. lavendulae* 9000, (g) *S. lavendulae* 3440-8, (h) *S. lavendulae* 3531.

Both strains were found to be indistinguishable from another strain of *S. albus* (B1337) and morphologically and serologically unlike a second strain of *S. erythreus* (8594). It was therefore concluded that the first culture labelled *S. erythreus* was in fact a strain of *S. albus* and was wrongly labelled in our collection.

Although the species were clearly differentiated according to the number of lines produced with a specific antiserum, the five strains of *Streptomyces lavendulae* were difficult to separate owing to marked cross-reactions; this is illustrated in Fig 2(b). Many strong precipitation lines, some of which are confluent, are seen between three *S. lavendulae* strains and the antiserum prepared against one of them. Only two faint lines were produced with this serum and a different species, *S. fradiae*. The cross-reacting strains of *S. lavendulae* do not show any obvious relationships to the groupings of the strains of this species proposed by Okami (1956).

Application to new isolates

The immunodiffusion method has been used on several occasions to assist in the identification of antibiotic-producing streptomycetes isolated from soil. Isolate A9257, which produces the antibiotic antimycin, did not give any precipitation lines when tested against antisera to *Streptomyces* sp. 2288, another antimycin-producing strain (Leben & Keitt, 1948). This new isolate A9257 was later shown to differ in morphology and physiological reactions from *Streptomyces* sp. 2283.

Another *Streptomyces* isolate, which produced an antibiotic similar to antimycin in a preliminary characterization, gave a strong pattern of identity when tested by the gel diffusion method against *Streptomyces* sp. 2288 antiserum and the homologous antigen preparation; the isolate was shown to be very similar to 2288 when grown on the usual identification media.

Isolate B4049 was found to produce antibiotics very similar to if not identical with the neomycin complex. Morphologically this isolate resembled *Streptomyces albogriseolus*, a species previously reported to produce neomycin (Benedict *et al.* 1954). *S. albogriseolus* antisera gave six precipitation lines with the homologous antigen and six lines with isolate B4049. The close serological relationship between these two strains was emphasized by the confluence of at least four of these lines; the two other lines were weaker and did not extend far enough to give evidence of confluence. However, when the Jennings triangular plate method (Jennings & Malone, 1955) was used, seven lines were observed for each antigen and each line was seen to be confluent. The two strains were therefore indistinguishable by this method. Further verification of their immunological identity was obtained by antibody absorption tests. Another isolate L1652, which produced neomycin, gave five precipitation lines against *S. albogriseolus* antiserum and none against antiserum to *S. fradiae*, a streptomycete used to produce neomycin commercially. Further evidence for the use of this technique to compare antibiotic-producing strains was reported by Cross (1962) for isolates producing oxytetracycline.

DISCUSSION

Because of their technical simplicity and ability to distinguish between antigenic molecules, immunodiffusion techniques are finding increased application to the taxonomy of microorganisms. To our knowledge, however, only three reports of the double-diffusion technique being used with *Streptomyces* have appeared. Wodehouse & Backus (1957) briefly reported antiserum to *Streptomyces aureofaciens* which produced nine precipitation lines when tested against homologous antigen, and suggested that the method is well adapted for use as an aid in differentiating species and strains within the genus. Bunch & Barth (1958), during a study on streams polluted by fermentation wastes, found that the presence of *Streptomyces* could be detected immunologically and that at least five antigens could be detected in undisrupted *S. lavendulae* mycelium. Guthrie, Roach & Ferguson (1962) have given preliminary details of the methods they have used in their work on the serology of aerobic aquatic actinomycetes.

In the work reported here we have shown that double-diffusion techniques have considerable value, not only for identifying Streptomycetes but for supplying supplementary characteristics to aid in the classification of this complex group of micro-organisms. A considerable degree of species specificity has been shown to exist. It is also possible that the technique may differentiate between the strains of some species (Cross, 1962). The antigens common to many species, which have caused difficulties in the application of conventional agglutination and precipitation tests, are also apparent.

The problem of deciding what weight should be given to antigenic structures in the application of immunodiffusion to *Streptomyces* taxonomy, as compared to

morphology and physiology, must await the results of a greater number of comprehensive studies as Shattock (1955) indicated in connexion with the application of serological techniques to bacterial taxonomy. It would also seem worth while to explore the use of isolated cell-wall antigens, following the work of Cummins (1962) on related genera, in an attempt to decrease the number of cross-reactions. From the more practical viewpoint, since the double diffusion method can easily and quickly show similarities between species, it seems that it may be useful in the identification of isolates producing new antibiotics. Ideally a bank of reference antisera would be needed, but, since activity is lost on prolonged storage and the production of a large number of sera is expensive in terms of time and materials, this may not be a practical procedure. Nevertheless, suitable antiserum to a new isolate may be produced in under 6 weeks. This can then be tested against type cultures and a relatively rapid serological identification of the new isolate obtained while more extensive conventional studies are being undertaken.

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