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Extracellular Antigens of *Micropolyspora Faeni* Grown in Synthetic Medium

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Extracellular antigens were prepared by growing *Micropolyspora faeni* in a new synthetic medium (SMSL), and this antigenic preparation was compared with antigens obtained by the double-dialysis method. These two preparations were tested against pooled sera from farmer's lung patients and rabbits immunized with these antigens by the agar-gel double-diffusion, immunoelectrophoresis, and two-dimensional immunoelectrophoresis methods. The immunological cross-reactivity between *M. faeni* strains of diverse origin and between other species of thermophilic actinomycetes associated with hypersensitivity pneumonitis was also investigated. The usefulness of two-dimensional immunoelectrophoresis in the comparison and standardization of antigens is discussed.

Micropolyspora faeni, a thermophilic actinomycete frequently seen in moldy hay, is considered the most important causative agent of farmer's lung disease (3, 14). Antibodies against this organism have been detected by the agar-gel double-diffusion (DD) method in 75% of the patients with farmer's lung, in 20% of other cases, and in healthy individuals who had been exposed to the same environment (9).

The diagnosis of this hypersensitivity lung disease is based on the clinical findings and the demonstration of specific antibodies to any of the thermophilic actinomycetes, particularly against *M. faeni*. Although several serological tests such as DD, counter immunoelectrophoresis, hemagglutination, complement fixation, and indirect immunofluorescence are available to detect specific humoral antibodies against *M. faeni*, none has received general acceptance (8). Similarly, no general agreement exists on the best method of extracting antigens for serological purposes from the cultures of the organism. Previous reports show that antigen preparations from *M. faeni* showed as many as 75 different components and, at times, antibody response in patients may be directed to only a few (9, 17). Another possibility for the variable antibody response is that the antigen preparations may vary in their constituents from strain to strain and even from different batches of antigens from the same strain used for the tests (9, 15).

The results of earlier reports on the serological studies of hypersensitivity pneumonitis cannot be compared due to the diverse methods followed in the extraction of antigens used. Kobayashi et al. (10) extracted defatted moldy hay with 10% trichloroacetic acid, whereas Pepys et al. (14) extracted with 0.5% phenol saline. After *M. faeni* was isolated in pure cultures, solid- and liquid-media cultures were used in the preparation of antigens. However, in all these reports, complex media were used to grow the organism; thus media proteins were mixed with the antigens. To eliminate complex media content from the antigens, Edwards (5, 6) used a double-dialysis method. By this method, high-molecular-weight components are retained in the dialysis tubing while the organism is grown in the outer phase. By a second dialysis, all the media are eliminated from the extracellular metabolic antigens. However, our experience with a modified double-dialysis method (12) was not altogether satisfactory. It was found that media components formed a major portion of the antigens isolated; this may be due to the change in pore size of the dialysis tubing during autoclaving, to the destruction of the membrane by the growing organism, or a combination of both factors.

The object of the present study was to develop a synthetic medium to grow *M. faeni* and to investigate the optimum conditions of growth for extracellular antigen production. It was also our intention to compare extracellular antigens from synthetic medium with double-dialysis antigens and to study the cross-reactivity between different strains of *M. faeni*.

MATERIALS AND METHODS

Organisms. Four strains of *M. faeni* were used in the study. One strain (T-150) was received from the

American Type Culture Collection (ATCC 15347) and one each (T-154 and T-181) from J. Lacey (A-947), Rothamstead Experiment Station, England, and H. Lechevalier (V-4066), Rutgers University. The other strain (T-211) was isolated from humidifier debris from a patient's home. The identity of all strains was checked by the scheme of Kurup and Fink (11).

Conditions of growth. In a preliminary study, it was found that all the strains of *M. faeni* grew in a synthetic broth (AOAC, Difco) enriched with additional vitamins and spermidine. The composition of the medium is given in Table 1. The following sugars, namely, glucose, maltose, sucrose, lactose, mannitol, arabinose, and rhamnose, were added to the basal medium individually in various combinations, and the growth of all strains was studied. The medium pH was adjusted to 5.5, 6.5, 7.2, and 8.0 and the optimum for best growth was studied. Similarly, the ideal temperature of incubation, of four incubation temperatures, namely, 37, 42, 50, and 55°C, was also determined for all the different strains. Lastly, the effect of shaking of cultures for better growth and antigen production was studied in comparison with stationary cultures.

TABLE 1. *Synthetic medium (pH 7.2) for Micropolyspora faeni*

Constituent	Wt (g)
L-Cystine	0.05
DL-Methionine	0.37
L-Arginine	0.4
DL-Histidine	0.3
L-Lysine	0.85
L-Tyrosine	0.21
DL-Threonine	0.5
DL-Valine	1.0
L-Leucine	0.8
DL-Isoleucine	0.44
Amino acetic acid	0.06
DL-Serine	0.61
DL-Alanine	0.43
L-Glutamic acid	1.3
L-Aspartic acid	0.45
DL-Phenylalanine	0.26
DL-Tryptophan	0.05
L-Proline	0.05
Sodium chloride	3.0
Potassium chloride	0.2
Magnesium sulfate anhydrous reagent	0.05
Potassium phosphate	1.5
Disodium phosphate	4.0
Thiamine hydrochloride	0.01
Nicotinamide	0.011
Choline chloride	0.001
Folic acid	0.001
Inositol	0.002
Calcium pantothenate	0.001
Pyridoxal	0.001
Riboflavin	0.0001
Lactose	10.0
Spermidine	0.015
Distilled water	1,000 ml

Growth curve and antigen production. *M. faeni* T-150 was grown in AOAC broth with vitamins, spermidine, and lactose (SMSL) in 500-ml flasks at 50°C under continuous shaking. Cultures were removed every 24 h and killed and the organisms were filtered, washed several times, and then dried for constant weight. The filtrate was processed as described below, and the freeze-dried antigens were weighed. Two separate estimations were made and the average was taken.

Double-dialysis antigens. Antigens were prepared according to the method of Edwards (6), except that Trypticase soy broth (TSB; BBL) was used instead of nutrient broth and dialyzing tube (D-32, 7/8 inch [ca. 2 cm]; Arthur Thomas) was used instead of Visking dialysis tubing (12).

Antigens from *M. faeni* grown in synthetic medium. Strains of *M. faeni* were grown in SMSL medium at 50°C with continuous shaking at 200 rpm. A heavy suspension of the organisms in physiological saline was inoculated into 125 ml of SMSL medium and incubated at 50°C on a continuous shaker for 3 days. One milliliter each of this growth was used to inoculate nine flasks of SMSL medium for antigen preparation. After 5 to 7 days of incubation, the organisms were killed with 0.5% phenol. The broth was separated by filtration, and the filtrate was dialyzed against distilled water for 24 to 48 h. The dialysate was centrifuged at 10,000 rpm for 30 min. The supernatant was filtered through a membrane filter (450-nm pore size, Millipore Corp.). The filtrate was freeze-dried and reconstituted when needed.

Farmer's lung sera. Five sera from patients giving positive precipitin reactions to *M. faeni* by the DD were pooled and tested against various antigens by the serological methods described below.

Immune rabbit sera. Four white rabbits were injected subcutaneously with 10 mg of antigen in 1 ml of saline mixed with equal amounts of Freund incomplete adjuvant. Four to six injections were given at weekly intervals. The rabbits were bled 10 days after the last injection and serum was evaluated for antibody production by the DD method. Sera from rabbits with demonstrable antibody were pooled and used in the serological tests.

DD. DD studies were performed by a micromethod reported previously (2, 7). Double-dialysis antigens (DDA) were used at 30 mg/ml, and synthetic medium antigens (SMSLA) were used at 5 mg/ml for the DD test.

Immunoelectrophoresis. Immunoelectrophoresis was carried out on projection slides (8 by 10 cm). Clean, dry slides were coated with 15 ml of 1% agarose in Veronal acetate buffer (0.05 M, pH 8.6) containing 0.1% sodium azide. Five microliters (100 μg of protein) of antigens was subjected to electrophoresis for 3.5 h at 50 mA and 60 V per plate. After electrophoresis, strips moistened with antisera were placed between the migration path of antigens and allowed to diffuse for 24 to 48 h in a moist chamber.

Two dimensional immunoelectrophoresis. The method used is a modification of the one described by Thirkill and Kenny (16). Slides were coated, and antigens were subjected to electrophoresis as in the

case of immunoelectrophoresis, but for 4 h at 30 mA and 72 mV. After electrophoresis, the gel was removed from plates, leaving a 2-cm strip of antigen, and was replaced with 9.4 ml of agarose gel containing 5 to 10% immune sera. The concentration of sera used for each antigen was determined empirically. The plates were subjected to electrophoresis at right angles to the original direction at 35 mA and 80 mV for 4 h. The plates were then removed, and the precipitin lines were allowed to develop in a moist chamber at room temperature overnight.

All agar-gel slides and plates were stained with Coomassie brilliant blue, according to the method of Axelsen and Bock (1).

Cross-reactions. Cross-reactivity between strains of *M. faeni* and between DDA and SMSLA were studied by two-dimensional immunoelectrophoresis. Antisera were run against homologous and heterologous antigen preparations, and the precipitin profiles were compared.

Chemical analysis of antigens. Total protein in the antigens was estimated according to the method of Lowry et al. (13), and total carbohydrates were determined by the phenol-sulphuric acid method of Dubois et al. (4).

RESULTS

Physiochemical conditions of growth. All strains of *M. faeni* studied grew in the synthetic medium (basal medium), but growth was far less than was observed in TSB, as studied by dry weight. However, all four strains tested showed very good growth, equal to TSB or better, in SMSL. It was found that a medium pH of 7.2 and incubation temperatures of 50 to 55°C gave the best growth for all strains tested. Shaker cultures gave more growth in a shorter period of time than stationary cultures.

Growth curve and antigen production. Antigen production corresponding to the growth curve of *M. faeni* T-150 is shown in Fig. 1. The maximum growth in SMSL, as well as the maximum amount of antigens, was produced on day 7 of the incubation period. On day 5 and thereafter, the antigens showed good reactivity against antisera. The number of major antigenic peaks gradually increased from day 2 and thereafter and reached the maximum on day 5; thereafter, no appreciable change, except in intensity, was noted (Fig. 2 and 3).

Comparison of DD antigens and SMSLA. The major characteristics of these two antigenic preparations from *M. faeni* T-150 are given in Table 2. The extracellular antigens produced in SMSL medium showed 70 to 80% protein content versus 50% shown in antigens produced by the double dialysis method. The carbohydrate content of the SMSLA preparations varied from 12 to 15%, whereas 25% was detected in the DDA preparation. The yield of antigens also varied when the organism was grown by these two methods. The double dialysis method yielded 0.2 to 0.25 mg of extracellular antigens per ml of medium in 2 to 3 weeks of incubation, as against 0.5 to 1 mg/ml in SMSL medium after 5 to 7 days of incubation.

The SMSLA were used in a concentration of 5 mg/ml, compared with 30 mg of DDA per ml. The optimum concentrations of each antigen for obtaining a sharp and intense precipitin arc at the equivalent zone with the pooled farmer's lung and rabbit antisera were determined by trial and error. Both antigens reacted identically with selected farmer's lung and rabbit

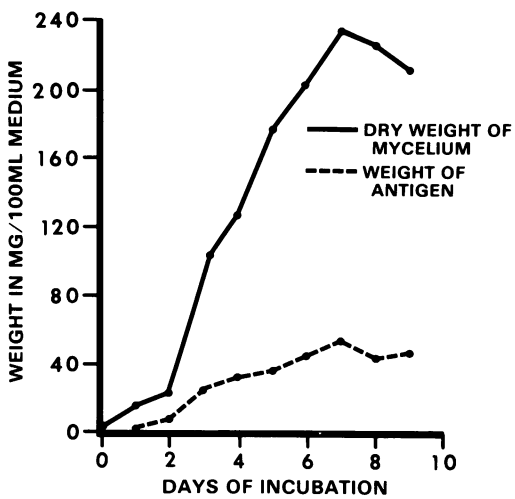

FIG. 1. *Dry weight of the organism versus extracellular antigens produced by M. faeni T-150 grown in SMSL medium under continuous shaking.*

TABLE 2. *Comparison of SMSLA and DDA*

Antigen	Characteristic					
	Incubation of culture (days)	Yield/100 ml of medium (mg)	Protein content (%)	Carbohydrate content (%)	Concn (mg/ml) used in:	
					DD test	Immunoelectrophoresis
SMSL	5-7	50-100	70-80	12-15	5	30
DD	14-21	20-25	50	25	30	80

sera against *M. faeni* by the DD method. No false-negative or false-positive reaction was detected when 24 positive and 15 negative control sera were tested with both antigen preparations. Both antigens also reacted strongly to the rabbit antisera produced by immunization with either antigen. Antibodies produced against one antigen preparation could not be completely absorbed by the other preparation, indicating that each preparation has its own unique antigenic determinants, as well as common determinants. These two antigens showed arcs of identity, partial identity, and nonidentity in DD. The major precipitin arcs were found to be common to both antigens by the immunoelectrophoresis and by the two-dimensional immunoelectrophoresis methods. However, these precipitin arcs, representing antigenic fractions, varied in the two antigen preparations, as evidenced by the size and density of the peaks. Approximately 24 precipitin arcs were determined when SMSLA reacted with homologous rabbit antibody (Fig. 4), as against 17 arcs formed by DDA with the homologous antibody (Fig. 5). All seven major peaks were also recognizable when these antigens were allowed to react with heterologous antisera (Fig. 6 and 7). Rabbit antisera against SMSLA, when absorbed with DDA, gave 8 to 10 precipitin arcs with SMSLA. On the other hand, six precipitin arcs were demonstrable when DDA reacted with homologous antibody absorbed with SMSLA.

The cross-reactivity between various strains of *M. faeni* is shown in Fig. 8 and 9. There is considerable variation between strains with regard to the number and intensity of precipitin peaks by the two-dimensional immunoelectrophoresis. However, antigens from all these strains reacted with pooled farmer's lung sera

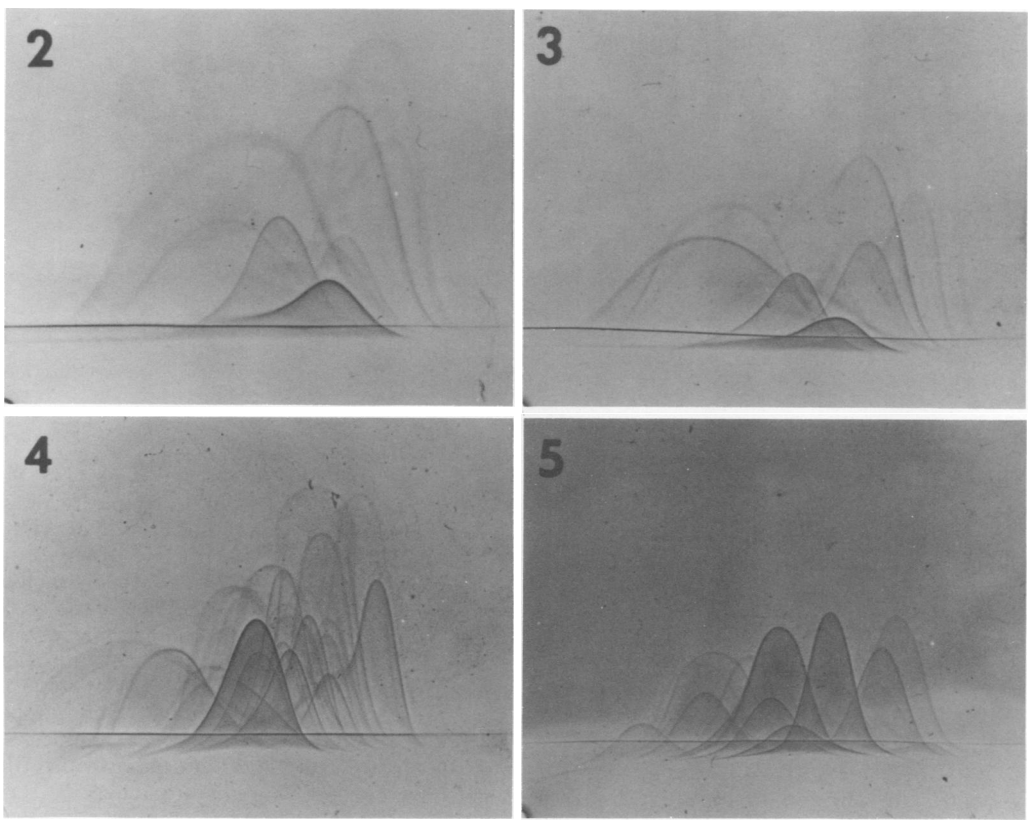

FIG. 2. *Two-dimensional immunoelectrophoretic profile of M. faeni T-150 antigens produced in SMSL medium after 3 days against anti-M faeni T-150 rabbit serum.*

FIG. 3. *Same as Fig. 2, except that the antigens were obtained from a 7-day-old culture.*

FIG. 4. *Two-dimensional immunoelectrophoretic profile of SMSLA of M. faeni T-150 against homologous rabbit antisera.*

FIG. 5. *Two-dimensional immunoelectrophoretic profile of DDA of M. faeni T-150 against homologous rabbit antisera.*

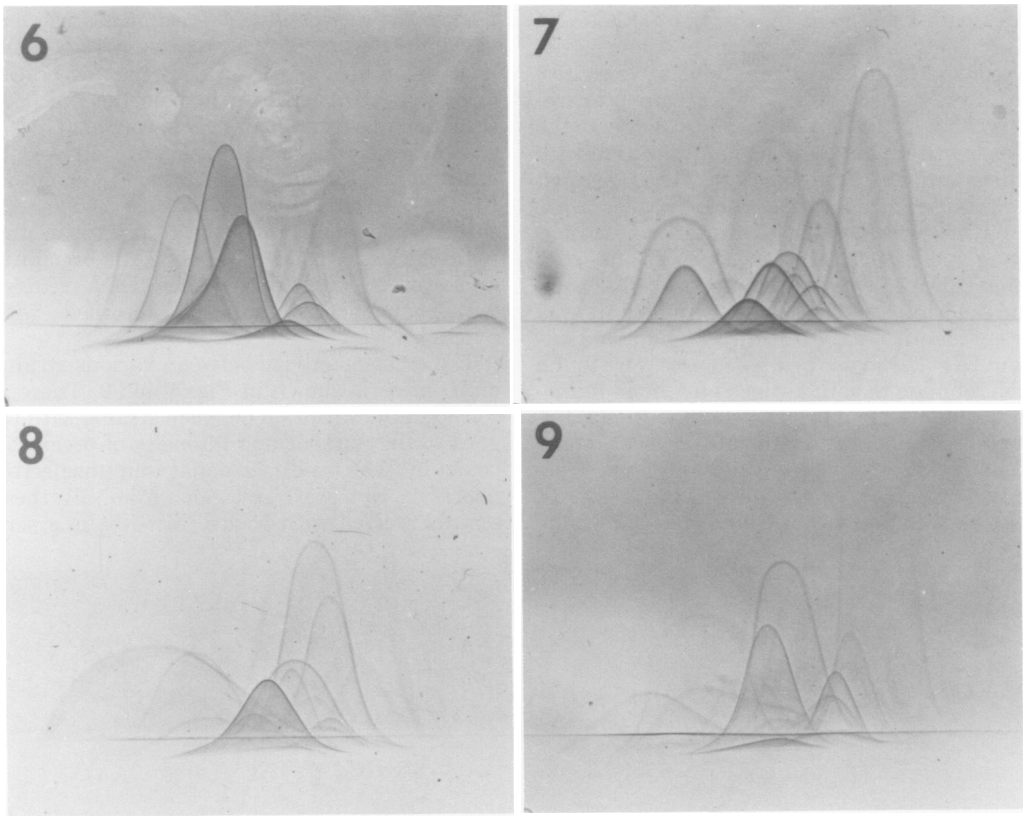

FIG. 6. *Two-dimensional immunoelectrophoretic profile of SMSLA (T-150) against rabbit antisera produced against DDA (T-150).*

FIG. 7. *Two-dimensional immunoelectrophoretic profile of DDA (T-150) against rabbit antisera produced agains SMSLA (T-150).*

FIG. 8. *Antigens from M. faeni T-154 were run against anti-rabbit sera produced by immunizing with SMSLA (T-150).*

FIG. 9. *Same as Fig. 8, except antigens from M. faeni T-181 were used.*

and gave 7 to 10 major precipitin peaks. Both SMSLA and DDA failed to react with sera from rabbits immunized with other thermophilic actinomycetes associated with hypersensitivity pneumonitis, namely *Thermoactinomyces candidus, T. vulgaris, T. sacchari,* and *Saccharomonospora viridis*.

DISCUSSION

This study indicates that the antigens produced in SMSL medium constitute a more reactive and pure antigen preparation than the DDA. Edwards (6) noticed that DDA was superior to other antigenic extractions such as water, sodium chloride, phenol, toluene, or ultrasonic disintegration followed by water extraction. In the present study, it was found that the SMSL antigens are superior to DDA in several respects. Inasmuch as the basal medium is commercially available, it is simple to make. By this method, a greater quantity of antigen is obtained in a shorter incubation time. As all the constituents of the medium are low-molecular-weight compounds (<500 molecular weight), the metabolic antigens can be separated free of medium. The physicochemical factors responsible for the variability of the antigen preparations such as pH, aeration, temperature of incubation, and chemical composition of the medium can be minimized by controlling these factors, and this can be achieved better in a defined, rather than in a complex, medium. Although both SMSLA and DDA reacted similarly against patients' sera, the fact that SMSLA were needed only in lower concentrations for serological tests indicates that this antigen preparation is superior to the DDA preparation.

The yield of antigen materials reported by Edwards (5, 6), by the double-dialysis method using 8 liters of nutrient broth, was only 1 g. He also failed to improve the yield of antigens by aerating the cultures. However, our results with the double-dialysis method indicate that the yield was more than doubled when TSB was used instead of nutrient broth. More strikingly, the SMSL medium gave a twofold or more increase in the antigens in a shorter incubation time than the double-dialysis method.

The number of precipitin arcs, particularly the weaker arcs, varied between antigens from different strains of *M. faeni* when two-dimensional immunoelectrophoresis was carried out against immune rabbit sera. Moreover, the total number of precipitin arcs did not exceed 24 in any of the systems investigated in the present study. Fletcher et al. (9) reported 29 separate precipitin arcs by DD, using farmer's lung sera and metabolic antigens from *M. faeni*. Our methods could not demonstrate, with certainty, more than six to eight precipitin arcs. Walbaum et al. (17) reported 75 bands by two-dimensional immunoelectrophoresis when purified rabbit antibody was used against *M. faeni* antigens. The low number of precipitin lines in the present study may be due to the use of crude antisera instead of the concentrated pure antibody used by those investigators.

The antigens obtained from *M. faeni* grown in SMSL medium were found to be superior to other antigenic preparations in purity, due to the exclusion of all media components. Two-dimensional immunoelectrophoresis is a good and reliable method by which antigenic components, present in different batches of antigens, can be compared. It is essential that, until purified fractions of antigens are available for serological studies, some quality assurance should be maintained to ascertain the reproducibility of the antigen preparations; this can be done by the two-dimensional immunoelectrophoresis.

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