Serological Relationships of *Corynebacteria*

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SUMMARY

The serological relationships between 39 strains of pathogenic corynebacteria from man, animals and plants were studied by precipitin, double gel-diffusion, and immuno-electrophoretic techniques, with polysaccharides and nucleoproteins as antigens. Five distinct serological groups were established among the plant pathogenic corynebacteria: (1) *Corynebacterium flaccumfaciens*, *C. flaccumfaciens* var. *aurantiacum*, *C. poinsettiae* and *C. betae*; (2) *C. michiganense* and *C. insidiosum*. (3) *C. tritici* and *C. rathayi*; (7) *C. sepedonicum*; and (5) *C. facians*. *C. diphtheriae* (type *gravis*, *mitis*, *intermedius*, and atypical forms), *C. hoffmanii*, (syn. *C. pseudodiphtheriticum*) and the animal pathogens *C. equi*, *C. renale* and *C. kutscheri* belonged to two or three serological groups. The plant pathogen *C. fascians* appeared to occupy an intermediate position between the plant and animal pathogens, and shared many antigens in common with the plant pathogens in group 1 and the animal pathogens. Other cross-relationships were detected between the groups. An immuno-electrophoretic study was made of protein fractions from the plant pathogenic bacteria.

INTRODUCTION

Studies of the serological relationships between various species of the genus *Corynebacterium* are few; most have been concerned with a limited number of species from only a few habitats. Rosenthal & Cox (1953, 1954), in their detailed study of four plant pathogenic species and one species from soil emphasized the desirability of a serological comparison between coryneform bacteria from plant and animals. Mushin, Naylor & Lahovary (1959) studied some of the *Corynebacterium* species from plants, animals and man with the object of using serology as a diagnostic tool for the plant pathogens, rather than as a method for investigating their inter-relationship and their relation to corynebacteria from other sources. Cummins & Harris (1956, 1958), Cummins (1962*a, b*) and Perkins & Cummins (1964) established relationships between several *Corynebacterium* species by comparing the antigens and composition of their cell walls. The work of Barber *et al.* (1963, 1965*a, b*, 1966) and Saragea, Barber, Meitert & Maximesco (1963) with *Corynebacterium diphtheriae* and with some corynebacteria from animals and plants indicated the importance of detailed serological studies in understanding the relations within the group of mammalian pathogens comprising the typical and atypical forms of *C. diphtheriae*, *C. hoffmanii* (syn. *C. pseudodiphtheriticum*) and *C. xerosis*, and revealed some of the serological relationships between these organisms and the animal and plant pathogenic corynebacteria.

Relationships within the corynebacteria have also been demonstrated by other

methods. Robinson (1966) investigated the esterase, catalase and peroxidase activity, and protein components of several coryneform bacteria by starch-gel electrophoresis, and established a relationship between the human, animal and plant pathogens, which enabled him to divide these organisms into groups and subdivisions. Ramamurthi (1959) made a comparative study of morphological and physiological characters of some coryneform plant pathogens and of single isolates of *Corynebacterium creatinovorus*, *C. equi* and *C. renale*. An Adansonian study of some corynebacteria and related organisms by Harrington (1966), who used a wide range of biochemical and physiological characters, showed that some of the organisms need to be reclassified. Lelliott (1966) pointed out that further morphological, physiological and serological work is needed to confirm his suggestions on the division of plant pathogenic coryneforms and their relation to other corynebacteria.

The work presented in this paper was done with a view to extending the understanding of the serological, and perhaps taxonomic, relationships between coryneform bacteria from plants, animals and man, by means of precipitin, double gel-diffusion and immuno-electrophoretic techniques.

METHODS

Strains examined. Thirty-nine strains of various *Corynebacterium* species were used; ten species were from plants, three from animals and three from man. These cultures were obtained from various foreign and local collections (Table 1).

Production of antisera. The technique used to obtain antisera with high antibody titres was that described by Barber *et al.* (1963, 1965*a*) and Saragea *et al.* (1963), with slight modifications. Briefly this technique consists of the hyperimmunization of rabbits with cultures in Freund incomplete adjuvant: lanolin, 20 ml.; paraffin oil, 40 ml.; bacterial suspension at $1.6-2.0 \times 10^9$ bacteria/ml., 40 ml. For plant pathogenic corynebacteria, living suspensions from 48 hr cultures on agar slopes (corn-steep, 1.5 % (w/v); peptone, 0.5 %; glucose, 1 %; NaCl, 0.5 %; CaCO_3 , 0.05 %; distilled water, 100 ml.) were used, and for animal and human corynebacteria, suspensions of 24-hr cultures on meat broth agar slopes were used. Four ml. of antigen suspension were injected into rabbits (2.8-3.0 kg.) subcutaneously into the nape region three times at 21-day intervals. Twenty-one days after the third inoculation of antigen with Freund adjuvant, five or six intravenous inoculations were given at 6 to 7-day intervals with nucleoprotein extracts, in increasing doses from 1 to 5 mg. When the test bleeding did not give a satisfactory precipitin reaction in tubes with homologous polysaccharide, immunization was continued for another two or three intravenous injections.

Production of antigens. Nucleoprotein and polysaccharide antigens were used. They were obtained by extraction of acetone-dried bacteria by the technique described by Barber *et al.* (1963, 1965*a*) and Saragea *et al.* (1963). Cultures were grown on the media described above in *Production of antisera*. To obtain nucleoprotein extracts, 5 g. of dry bacteria were suspended in 80 ml. 2 % (w/v) sodium deoxycholate solution for 24 hr at 37°. After centrifugation the supernatant fluid was dialysed for 4-5 days against running tap water, followed by centrifugation of the dialysis residue and dialysis of the supernatant fluid for 2-3 days against distilled water at refrigerator temperature, further centrifugation and finally sterilization by filtration. For polysaccharide extracts, the sediment resulting after treatment with sodium deoxycholate

solution was resuspended in 50–60 ml. of 0.3 M-HCl and kept for 45 min. at 90° in a water bath with continuous stirring. After centrifugation or filtration, the supernatant fluid or filtrate was treated with trichloroacetic acid (final concentration 10% w/v). The resultant precipitate was removed by filtration and the filtrate dialysed for 3 days in running tap water and for 2 days in distilled water at refrigerator temperature.

Gel diffusion and immuno-electrophoresis. Double gel-diffusion was done by the Ouchterlony technique (1949, 1958) and immuno-electrophoresis as shown in detail in another paper (Lazar, 1967), generally by using the techniques described for

Table 1. *Corynebacterium* species studied

Species and culture no.	Source	Obtained from:
<i>C. diphtheriae</i> type <i>gravis</i> 4895, 5429	Man	'Dr I. Cantacuzino', Institute, Bucarest
<i>C. diphtheriae</i> type <i>mitis</i> 411		
<i>C. diphtheriae</i> P.W.8.		
<i>C. diphtheriae</i> type <i>intermedius</i> 1180,		
3522, 3536, 4109, 4465		
<i>C. diphtheriae</i> , atypical form 4409		
4528, 4852		
<i>C. hoffmanii</i> 4855	Animals	Faculty of Vet. Med., Bucarest
<i>C. equi</i> 4031		
<i>C. renale</i> 2276		
<i>C. kutscheri</i> 239	Plants	NCPPB* NCPPB Ottawa, Canada NCPPB NCPPB Our own isolation NCPPB Leningrad, U.S.S.R. Quebec, Canada NCPPB Quebec, Canada NCPPB NCPPB NCPPB NCPPB Ottawa, Canada NCPPB Ottawa, Canada
<i>C. betae</i> 373, 375		
<i>C. flaccumfaciens</i> 559, 567		
<i>C. flaccumfaciens</i> 706		
<i>C. f. var. aurantiacum</i> 558		
<i>C. poinsettiae</i> 845, 848		
<i>C. michiganense</i> 1		
<i>C. michiganense</i> 515		
<i>C. michiganense</i> 201		
<i>C. michiganense</i> 704		
<i>C. insidiosum</i> 83		
<i>C. insidiosum</i> 702		
<i>C. tritici</i> 255, 471		
<i>C. rathayi</i> 797		
<i>C. sepedonicum</i> 299		
<i>C. sepedonicum</i> 708		
<i>C. fascians</i> 156, 469		
<i>C. fascians</i> 716		

* National Collection of Plant Pathogenic Bacteria, Harpenden, Herts, England.

proteins from human serum (Grabar & Williams, 1953; Grabar & Burtin, 1960; Heremans, 1961; Bratu, 1962). The gel was prepared by dissolving 1.2% of Noble agar (Difco) in veronal buffer (veronal, 2.0766 g.; medinal, 13.4170 g.; sodium acetate, 1.2952 g.; potassium oxalate, 0.8346 g.; distilled water 2 l.; pH 8.6). The best results were obtained when 13 × 18 cm. glass plates were covered with 40–45 ml. gel. For nucleoprotein migration a series, each of two plates, was introduced into the electrophoresis bath (which contained veronal buffer) at a working potential of about 250–300 V, adjusted to an estimated 1.5 mA/cm. of plate. The experiment was carried out in an electrophoresis apparatus of the QE-201 type, manufactured in Hungary. Plates that showed a suitable immuno-electrophoretic reaction were stained with 'Amidoschwarz' solution (90% (v/v) methanol, 10% glacial acetic acid, 0.3%

'Amidoschwarz' prepared in a water bath for 4-6 hr. After staining, the plates were decolourized with a 2 % acetic acid solution, for one or several days, until the background of the plate was clean. The fractions represented by precipitation arcs along the migration axis were identified by comparison with those reported for fractions in human serum by Grabar & Williams (1953), Heremans (1961), Schmid (1961) and, Haggis *et al.* (1964).

RESULTS

The results of this study are presented in Tables 2-5 and partially illustrated in Figs. 1 and 2.

Tube precipitin reactions

Tube precipitin reactions (Table 2) indicated that only *Corynebacterium fascians*, among the plant pathogens, shared more than a limited amount of nucleoprotein antigen with *C. diphtheriae*. Lesser amounts were shared with *C. diphtheriae* by *C. poinsettiae*, *C. flaccumfaciens*, *C. betae*, *C. tritici* and *C. equi*, and little or none by *C. insidiosum*, *C. michiganense*, *C. sepedonicum*, *C. rathayi*, *C. renale* and *C. kutscheri*. The species from animals shared common antigens with *C. diphtheriae*, flagellate plant pathogenic corynebacteria and *C. fascians*.

Reactions within the plant pathogenic species suggested five serological groups: (1) *C. poinsettiae*, *C. flaccumfaciens*, *C. betae*; (2) *C. insidiosum*, *C. michiganense*; (3) *C. tritici*, *C. rathayi*; (4) *C. sepedonicum*; (5) *C. fascians*. Small amounts of common antigens were found between some species of these groups (Table 2).

Gel diffusion with nucleoprotein extracts

The results of the precipitation reaction by double gel-diffusion are shown in Table 3. The reactions between the antisera of the different types of *Corynebacterium diphtheriae* and the antigens of the phytopathogenic species indicated some relationship, expressed by weak or very weak precipitation lines, between *C. diphtheriae* and the following plant pathogenic species: *C. fascians*, *C. poinsettiae*, *C. flaccumfaciens*, *C. flaccumfaciens* var. *aurantiacum*, *C. insidiosum*, *C. michiganense*, *C. tritici*, *C. rathayi* and *C. sepedonicum*.

The antigens of species from animals did not react with the available diphtheriae antisera. However, positive reactions were obtained between the antigens (particularly the nucleoprotein antigens) of *Corynebacterium diphtheriae* and the antisera of the animal pathogens *C. renale* and *C. equi*.

Antigens of species from plants reacted positively with the antisera of species from animals, the more intense reactions being: *Corynebacterium betae*, *C. flaccumfaciens*, *C. poinsettiae*, *C. fascians* and *C. michiganense* with antiserum of *C. equi*; *C. insidiosum* and to a lesser degree *C. poinsettiae* and *C. flaccumfaciens* var. *aurantiacum* with antiserum of *C. kutscheri*; *C. flaccumfaciens* var. *aurantiacum*, *C. flaccumfaciens*, *C. poinsettiae* and *C. fascians* with the antiserum of *C. renale*.

The antigenic structural relationship between species from man and from plants was clearer when diphtheria antigens were tested with antisera for plant pathogens than when diphtheria antisera were used against antigens from plant pathogens. This was because in some cases higher-titre antisera were obtained for plant pathogenic species than for species from man. *Corynebacterium fascians* appeared to be more closely related serologically to the three types of *C. diphtheriae* than were the other

Table 4. Reactions obtained by double gel diffusion with polysaccharide extracts

The method used is the same as that described at the head of Table 3.

Antigens																	
<i>C. fascians</i> 469	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>C. fascians</i> 156	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>C. sepedonicum</i> 299	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>C. rathayi</i> 797	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>C. tritici</i> 471	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>C. tritici</i> 255	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>C. insidiosum</i> 83	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>C. michiganense</i> 704	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>C. michiganense</i> 201	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>C. betae</i> 375	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>C. flaccumfaciens</i> var. <i>aurant.</i> 558	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>C. flaccumfaciens</i> 559	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>C. poinsettiae</i> 845	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>C. kutscheri</i> 239	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>C. renale</i> 2276	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>C. equi</i> 4031	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>C. diphtheriae</i> , atypical form 4852	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>C. diphtheriae</i> interm. 3536	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>C. diphtheriae</i> interm. 3522	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>C. diphtheriae</i> interm. 4465	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>C. diphtheriae</i> mitis 411	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>C. diphtheriae</i> gravis 5429	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

plant pathogens. A positive reaction, though only with the *mitis* type of *C. diphtheriae*, was obtained with the antisera of the various strains of *C. michiganense*, *C. insidiosum* and *C. betae*. The nucleoprotein antigens of various strains belonging to atypical forms of *C. diphtheriae* also gave one or two precipitation lines with the antisera of *C. flaccumfaciens* var. *aurantiacum* and *C. poinsettiae*, and one single and weak precipitation line with the antiserum of *C. michiganense* 704; a very weak or doubtful reaction was obtained with *C. michiganense* 1 and 515.

Some antisera of the plant pathogenic species formed a clear precipitation line with some antigens of different species from animals: *Corynebacterium insidiosum* with *C. equi* and *C. kutscheri*, *C. fascians* 469 with *C. kutscheri*; *C. sepedonicum* 299, and less clearly *C. michiganense* 704, with *C. renale*.

The same five groups within the plant pathogenic corynebacteria were distinguished by gel diffusion as were demonstrable by tube precipitin reactions (see above).

Gel diffusion with polysaccharide extracts

The results of double gel diffusion with polysaccharide antigens are given in Table 4. The weaker relationships between groups were less clear than with nucleoprotein extracts. Thus the antisera of *Corynebacterium flaccumfaciens* 599, *C. flaccumfaciens* var. *aurantiacum* 558, *C. poinsettiae* 848, *C. sepedonicum* 299, *C. michiganense* 705, *C. diphtheriae* type *mitis* 411, as well as a few others, gave no satisfactory reactions with homologous antigens, probably because these strains produced little polysaccharide or because what polysaccharide they did produce was serologically inactive. Generally the relationships demonstrated were the same as those with nucleoprotein extracts. Antisera of *C. diphtheriae* gave usually weak reactions with the antigens of one or more strains of *C. renale*, *C. kutscheri*, *C. poinsettiae*, *C. flaccumfaciens* var. *aurantiacum*, *C. betae* and *C. fascians*. Some of the antisera against species from animals reacted with antigens of one or more strains of *C. diphtheriae* type *intermedius*, *C. fascians*, *C. betae*, and *C. flaccumfaciens* var. *aurantiacum*. The antisera of plant pathogens reacted with some of the antigen preparations of *C. diphtheriae* type *intermedius*, *C. diphtheriae* type *gravis*, *C. renale* and *C. kutscheri*. The reactions between the polysaccharide antigens and the antisera of plant pathogenic species reinforced the serological groups of species determined by the reaction with nucleoprotein antigens (see above).

The interrelationships schematized in Fig. 2 show that plant pathogenic corynebacteria of groups 1, 2 and 5 share more polysaccharide antigens with the human and animal corynebacteria than do groups 3 and 4. *Corynebacterium fascians* (strain 469) is very rich in polysaccharide antibodies, as well as in nucleoprotein antibodies (Tables 3, 4) and shows a particularly clear relationship with the corynebacteria from man and animals and also with the other groups of plant pathogenic corynebacteria.

Immuno-electrophoresis

The results obtained by double gel diffusion were confirmed by immuno-electrophoresis. It was also possible by using immuno-electrophoretic techniques to identify and make a more exact estimation of the number of antigenic fractions.

Antisera with high antibody titres, and the application for migration of about 0.2 ml. of not less than 1.5–2.0 mg. nucleoprotein antigen/ml., were necessary to

Table 5. *Immuno-electrophoretic analysis of nucleoprotein antigens from Corynebacteria*

Fractions were separated in a QE-201 electrophoretic bath at 250-300 V. working potential and 1.5 mA. per cm. using a gel of veronal buffer, pH 8.6, and 1.2 % agar.

Antisera for	Nucleoprotein antigens from:																			
	<i>C. betae</i> 373					<i>C. poinsettiae</i> 845					<i>C. poinsettiae</i> 848					<i>C. flaccumfaciens</i> 567				
	O	A	α	β	γ	O	A	α	β	γ	O	A	α	β	γ	O	A	α	β	γ
<i>C. betae</i> 373	-	+	+	+	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+	-
<i>C. betae</i> 375	-	+	+	+	-	-	+	+	+	-	-	+	+	+	-	-	+	+	+	-
<i>C. poinsettiae</i> 845	-	-	+	+	+	-	+	+	+	+	-	+	+	+	+	-	+	+	+	+
<i>C. poinsettiae</i> 848	-	-	+	+	+	-	+	+	+	+	-	+	+	+	+	-	+	+	+	+
<i>C. flaccumfaciens</i> 567	-	+	+	+	-	-	+	+	+	+	-	+	+	+	+	-	+	+	+	-
<i>C. f. var. aurantiacum</i> 558	-	+	+	+	-	-	+	+	+	+	-	+	+	+	+	-	+	+	+	-
<i>C. michiganense</i> 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>C. tritici</i> 255	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>C. fascians</i> 469	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>C. renale</i> 2276	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Antisera for	Nucleoprotein antigens from:																			
	<i>C. rathayi</i> 797					<i>C. sepehdonicum</i> 299					<i>C. fascians</i> 156					<i>C. fascians</i> 469				
	O	A	α	β	γ	O	A	α	β	γ	O	A	α	β	γ	O	A	α	β	γ
<i>C. betae</i> 373	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>C. betae</i> 375	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>C. poinsettiae</i> 845	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>C. poinsettiae</i> 848	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>C. flaccumfaciens</i> 567	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>C. f. var. aurantiacum</i> 558	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>C. michiganense</i> 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>C. tritici</i> 255	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>C. fascians</i> 469	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>C. renale</i> 2276	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

O = Orosomucoids; A = albumens; α, β, γ = fractions whose position along the migration axis is similar to that of α, β and γ fraction in human serum. + = positive reaction; ± very weak or doubtful reaction; - no reaction.

detect and identify some of the antigens detectable by gel diffusion. These conditions were not realized with some of the nucleoprotein extracts, including those of *Corynebacterium sepedonicum* 299, *C. flaccumfaciens* 559, *C. insidiosum* 83, *C. rathayi* 797, *C. kutscheri*, *C. diphtheriae* (some types), etc., and some antisera, including those of *C. insidiosum*, *C. sepedonicum*, *C. fascians* (some strains), *C. kutscheri*, *C. equi*. For this reason some of the antigens and antisera tested are not included in Table 5.

The electrophoretic migration of the nucleoproteins was towards the positive pole and their deposition was in zones comparable to those of the orosomucoids, albumins, and group α and β fractions from human serum. In runs in which the nucleoprotein extracts of the different *Corynebacterium* species were compared with human serum, fractions with the same mobility as the α group and the albumins were detected most frequently; fractions comparable with the orosomucoids, and with group β less frequently. No fractions comparable to group γ were detected.

The species richest (5-8) in fractions identifiable with the proteins from human serum were: *Corynebacterium flaccumfaciens* var. *aurantiacum* 558, *C. poinsettiae* 845, 848, *C. flaccumfaciens* 567, 706, *C. betae* 373, 375, *C. fascians* 469, 156, and *C. michiganense* 1. A few comparable fractions (2-3) were identified in: *C. tritici* 255, *C. rathayi* 797, *C. fascians* 716, *C. diphtheriae* 5429, 411, 4465, 4528, *C. renale* 2276 and *C. kutscheri* 239. It is possible that, using more concentrated nucleoprotein antigens and antisera richer in antibodies, these strains and those of others including *C. sepedonicum*, *C. insidiosum* and *C. equi*, could be shown to have more protein fractions in the nucleoprotein antigens extractable from bacterial cells with sodium deoxycholate.

In addition to confirming the groups obtained by tube precipitin and gel-diffusion techniques, immuno-electrophoresis confirmed that group I was very rich in antigenic fractions and group 3 poor. It also indicated closer relationship between *Corynebacterium flaccumfaciens* var. *aurantiacum* and *C. poinsettiae* than between *C. flaccumfaciens* var. *aurantiacum* and *C. flaccumfaciens*. *C. flaccumfaciens* var. *aurantiacum* and *C. poinsettiae* have fractions identical in number and in their electrophoretic position.

Plant pathogenic corynebacteria from groups 1, 5 and 3 appear to be related to human and animal coryneforms in albumens and fractions from group α and possibly in fractions from group β .

DISCUSSION

By means of the classical methods and criteria used for differentiating diphtheroids, Lazar (1968) has shown that most of the plant pathogenic corynebacteria resemble the pseudodiphtheria organisms from man and animals in many of their characters. *Corynebacterium fascians* presented a striking resemblance to *C. diphtheriae* and particularly to its atypical forms; this is not in agreement with the suggestion that *C. fascians* should be classified as a member of the genus *Nocardia* (Ramamurthi, 1959) or that it is a boundary form between the genera *Corynebacterium*, *Mycobacterium* and *Nocardia* (Lacey, 1955).

The present serological study of a wide range of coryneform bacteria by precipitation-double gel-diffusion, and immuno-electrophoresis techniques has confirmed and amplified previous results obtained with agglutinins, cell extracts and cell-wall, composition (Mushin *et al.* 1959; Cummins, 1962*a*, *b*; Cummins & Harris, 1956, 1958; Perkins & Cummins, 1964; Barber *et al.* 1965*b*, 1966), and has indicated the

broad relationships demonstrated in Figs. 1 and 2. It appears that plant pathogenic coryneforms can be placed in five distinct serological groups: (1) *Corynebacterium flaccumfaciens*, *C. flaccumfaciens* var. *aurantiacum*, *C. poinsettiae*, *C. betae*; (2) *C. michiganense* and *C. insidiosum*; (3) *C. tritici* and *C. rathayi*; (4) *C. sepedonicum*; (5) *C. fascians*.

Fig. 1. Serological relationships as determined by double gel diffusion and immunoelectrophoresis techniques, using nucleoprotein extracts as antigen. —, strong reaction; ---, reaction; -.-, weak reaction;, very weak or doubtful reaction.

Fig. 2. Serological relationships as determined by double gel diffusion method, using polysaccharide extracts as antigen. —, Strong reaction; ---, reaction; -.-, weak reaction;, very weak or doubtful reaction.

The results have confirmed the work of Rosenthal & Cox (1953, 1954) who, by cross-absorption, showed serological identity between the *Corynebacterium michiganense* and the *C. insidiosum* AB serotypes, and the very close serological relationship of *C. poinsettiae* with *C. flaccumfaciens*. The results also confirm the finding by Katznelson & Sutton (1956) that *C. sepedonicum* shares one or more antigens with *C. michiganense* and *C. flaccumfaciens*, but also show that *C. sepedonicum* shares antigens with *C. poinsettiae*, *C. fascians*, *C. betae* and *C. rathayi*. The results also confirm the division of the plant pathogenic coryneform bacteria suggested by Lelliott (1966), but show that his first group contains three serological groups, and his second group two serological groups.

Corynebacterium species from plants, animals and man are antigenically related (Figs. 1, 2). *Corynebacterium fascians* and the species in the *C. flaccumfaciens* group (group 1) are the plant pathogens most closely related to corynebacteria from man and animals. *Corynebacterium fascians* appears to be most closely related to *C. diphtheriae*, and species in group 1 to the atypical forms of *C. diphtheriae*. *Corynebacterium fascians* appears to occupy a central position, showing very close affinity with the human and animal corynebacteria, and also with the flagellated group 1. Other approaches to the problem have also indicated this intermediate or central position of *C. fascians*. Robinson (1966) showed that *C. fascians* produced a pattern of enzymes similar to that produced by some plant, some human and some animal pathogenic corynebacteria. Its peroxidase pattern was similar to that of the plant pathogens but its esterase and catalase pattern was like that of the animal pathogens. Robinson also found that *C. tritici* had an enzyme pattern which showed very little relationship with the other plant pathogens; a similar lack of relationship in nucleoprotein antigens between *C. tritici* and *C. rathayi* and the other plant pathogenic corynebacteria is shown in Fig. 1. An Adansonian taxonomic study (Harrington, 1966) has also indicated that *C. fascians* appears to be the plant pathogen most closely related to the animal strains.

Barber *et al.* (1963, 1965a) and Saragea *et al.* (1963) showed that *Corynebacterium diphtheriae* and the other *Corynebacterium* species from man are related to each other only through their nucleoprotein antigens, and that their polysaccharide antigens are specific. They also showed a serological specificity for the polysaccharides isolated from different plant pathogenic corynebacteria (Barber *et al.* 1966). The results reported here, however, show that the polysaccharide antigens of several *Corynebacterium* species from plants *C. fascians* 469, 156; *C. betae* 375; *C. poinsettiae* 845; *C. flaccumfaciens* var. *aurantiacum* 558) react with *C. diphtheriae* antisera and, even more clearly, that the polysaccharides of *C. diphtheriae* react with the antisera of *C. fascians* 469, *C. insidiosum* 83, *C. kutscheri* 239 and *C. equi* 4031. Other examples of common polysaccharide antigens are given in Table 4.

The serological relationship found between certain species from plants and *Corynebacterium diphtheriae* (including its atypical forms) seems to be at least as close as that ascertained by Barber *et al.* (1963, 1965a) and Saragea *et al.* (1963) between *C. diphtheriae* on the one hand, and the atypical forms and *C. hoffmanii* and *C. xerosis* on the other. It appears justifiable to consider that, if the place of pseudodiphtheroids and atypical forms within the genus *Corynebacterium* cannot be doubted, then neither can that of the diphtheroids from plants among which *C. fascians* is included. As Gorlenko (1964) suggested, *C. fascians* may be an intermediary form in this group of

bacteria. To understand more clearly the interrelationships of corynebacteria, it will be necessary to study the relationship of members of the genus *Corynebacterium*, particularly *C. fascians* and *C. renale*, with representative species of the genera *Arthrobacter* and *Nocardia*.

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