# Cross-Reaction of *Corynebacterium sepedonicum* Antisera With *C. insidiosum*, *C. michiganense*, and an Unidentified Coryneform Bacterium

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## ABSTRACT

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The immunoglobulin G (IgG) component of antiserum produced in rabbits against glutaraldehyde-fixed cells of *Corynebacterium sepedonicum* was purified by column chromatography on Sephacryl S300 and used for indirect immunofluorescence staining. Specific activity (immunofluorescence titer per milligram of protein) of IgG fractions was determined with *C. sepedonicum*, *C. insidiosum*, *C. michiganense*, and an unidentified coryneform bacterium isolated from a potato stem. Specific activity with *C. sepedonicum* was greater than with the heterologous bacteria in antisera obtained up to 20 wk after beginning immunization, but thereafter was about equal to or less than the reaction with the cross-reacting species. An antigenic fraction was extracted from acetone-dried *C. sepedonicum* cells with hot phenol and antigenic activity was detected in culture filtrates by agar double diffusion. The culture filtrate but not the phenol extract sensitized sheep erythrocytes in indirect hemagglutination.

*Additional key words*: bacterial ring rot of potato, immunofluorescence.

Serological techniques are increasingly being recognized as alternative procedures to the Gram stain method for diagnosing and detecting bacterial ring rot of potato caused by *Corynebacterium sepedonicum* (Spieck and Kotth.) Skapt. and Burkh. (3,6,16,23,24). The accuracy and efficacy of these methods, however, are dependent on the specificity of the antisera being used. Antiserum specificity varies with bacterial strain or antigenic preparation used as immunogen and with individual animals used for antisera production. However, measurement of antisera specificity also has been variable and is a function of the serological method employed and on the number of organisms tested.

A limited number of relatively weak serological cross-reactions have been detected in *C. sepedonicum* antisera with cell agglutination (13) and latex agglutination (24) techniques. Additional serological cross-reactions of *C. sepedonicum* antisera with other Gram-positive and even some Gram-negative bacteria have been reported when sensitive immunofluorescence tests were used (6,21,26).

Serological cross-reactions are common among Gram-positive bacteria and are often due to common carbohydrate antigenic determinants on peptidoglycans (22), capsular polysaccharides (18), or teichoic acids (28). Although the common antigens may

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have taxonomic significance, they limit the usefulness of sensitive serological tests for pathogenic species.

In this study some cross-reactions of *C. sepedonicum* antisera are evaluated and a preliminary consideration of *C. sepedonicum* antigens is made.

## MATERIALS AND METHODS

**Bacterial cultures.** Strains of *Corynebacterium* spp. used in this study are given in Table 1. In addition, a nonpathogenic coryneform bacterium (strain A), isolated from a symptomless potato stem (5), was used. Cultures were stored on YGM slants (6) at 3 C and cultured on YGM medium or in YGM broth at 25–26 C.

**Antisera production and fractionation.** Strain CS3 was used as antigen for immunization. Seven-day-old cells were washed three times with sterile distilled water, suspended to $O.D._{660\ nm} = 1.0$ measured with a Spectronic 20 (Bausch and Lomb) spectrophotometer, fixed in glutaraldehyde (1), and stored at 4 C.

Two New Zealand white rabbits were each injected according to the same immunization schedule by using 0.5 ml of antigen emulsified with an equal volume of Freund's incomplete adjuvant for intramuscular injections and 0.5 ml of antigen without adjuvant for intravenous injections. Intramuscular injections were given on days 0, 9, 17, 24, and 151; intravenous injections on days 50 and 59. Rabbits were bled from the marginal ear veins.

Sera were fractionated on a Sephacryl S300 superfine (Pharmacia) 85 × 2.6-cm column. The eluant buffer was 0.1 M Tris HCl/0.5 M NaCl, pH 8.0, plus 0.01% $NaN_3$ and the flow rate was 24 ml/hr. Two milliliters of serum were applied to the column and 100 3-ml fractions were collected. Ultraviolet absorption was monitored at 280 nm by using an ISCO UA2 analyzer. Fractions in each 280-nm absorption peak were pooled for further analyses.

Protein concentrations were estimated from 280-nm absorption values by using 1.4 as the extinction coefficient.

**Serological techniques.** For indirect immunofluorescence, bacterial suspensions were washed twice with distilled water and resuspended in water to $O.D._{660\ nm} = 0.1$. These suspensions were diluted 1:10 and the cells in single drops were heat-fixed to the wells of microtiter slides. To each preparation a drop of antiserum, diluted in 0.01 M phosphate-buffered (pH 7.2) 0.85% saline (PBS) was applied. Slides were incubated in a moist chamber for 30 min, washed with PBS containing 0.5 ml of Tween-20 per liter, rinsed with distilled water, and blotted dry. A drop of antirabbit IgG antiserum conjugated with fluorescein isothiocyanate (Litton Bionetics, Kensington, MD 20795) diluted 1:40 in PBS was then applied to each preparation; slides were incubated another 30 min in a dark moist chamber, and washed as before. Coverslips (No. 1 size) were mounted with glycerol-PBS (10:1) and preparations were observed with a Zeiss photomicroscope equipped with an oil-immersion objective (×100), a III RS epi-illuminator, a 48 77 11 filter set, and an HBO 50 light source.

Specific activities of serum fractions were calculated in units expressed as the reciprocal of immunofluorescence titer per milligram of protein (20). The titers were measured as the highest

TABLE 1. Source of *Corynebacterium* spp.[a] cultures

| Species and Strains | Obtained from | Isolated by | Geographic origin |
|---|---|---|---|
| *Corynebacterium betae* | | | |
| ICPB CB101 | M. P. Starr | W. J. Dowson | England |
| ICPB CB103 | M. P. Starr | W. G. Keyworth | England |
| *C. fascians* | | | |
| ICPB CF17 | M. P. Starr | A. Davey | Ohio |
| ICPB CF107 | M. P. Starr | S. E. Jacobs | England |
| *C. flaccumfaciens* | | | |
| ICPB CF3 | M. P. Starr | H. Purdy | New York |
| ICPB CF8 | M. P. Starr | W. H. Burkholder | ... |
| *C. insidiosum* | | | |
| ICPB CI16 | M. P. Starr | ... | Kansas |
| ICPB CI102B | M. P. Starr | P. A. Ark | California |
| N | G. A. Nelson | C. D. Cox | ... |
| N53 | G. A. Nelson | C. D. Cox | ... |
| 77-2(3) | G. A. Nelson | C. D. Cox | Alberta |
| CI1 | R. J. Copeman | G. A. Nelson | Alberta |
| *C. iranicum* | | | |
| ICPB CI147 | M. P. Starr | E. Skarandi | Iran |
| ICPB CI148 | M. P. Starr | ... | ... |
| *C. michiganense* | | | |
| ICPB CM1 | M. P. Starr | W. H. Burkholder | New York |
| ICPB CM8 | M. P. Starr | W. J. Dowson | Kenya |
| CM9 | G. A. Nelson | ... | New York |
| CM3 | G. A. Nelson | G. A. Nelson | Alberta |
| *C. oortii* | | | |
| ICPB CO101 | M. P. Starr | H. P. Maas Geesteranus | Netherlands |
| ICPB CO102 | M. P. Starr | T. Kobayashi | Japan |
| *C. poinsettiae* | | | |
| ICPB CP2 | M. P. Starr | M. P. Starr | ... |
| ICPB CP109 | M. P. Starr | L. A. McFadden | Florida |
| *C. rathayi* | | | |
| ICPB CR1 | M. P. Starr | M. D'Oliveira | England |
| ICPB CR101 | M. P. Starr | M. Noble | England |
| *C. sepedonicum* | | | |
| ICPB CS5 | M. P. Starr | ... | New York |
| ICPB CS106 | M. P. Starr | J. G. Leach | West Virginia |
| CS3 | R. J. Copeman | G. A. Nelson | Alberta |
| CS15 | R. J. Copeman | S. A. Slack | Wisconsin |
| *C. tritici* | | | |
| ICPB CT102 | M. P. Starr | R. S. Vasudeva | India |
| ICPB CT104 | M. P. Starr | R. Amani | Iran |

[a] All species listed except *C. iranicum* and *C. tritici* are on the Approved Lists. It has been proposed that *C. iranicum* and *C. tritici* are pathovars of *C. michiganense* (8).

dilution at which individual cells were even barely visible.

Immunodiffusion was done with nonfractionated antiserum in plastic petri dishes (100 × 15 mm) containing 15 ml of an agar medium prepared with 0.8% Difco purified agar, 0.85% NaCl, and 200 ppm $NaN_3$. Wells 5 mm in diameter and 2 mm apart were cut in sets of six peripheral wells surrounding a center well.

For indirect hemagglutination, the method of Hewett et al (10) was used. Washed sheep red blood cells were mixed with several dilutions of test solutions to give a final 2% (v/v) suspension of red blood cells. After incubation of the mixture for 30 min at 37 C, the erythrocytes were washed three times in 0.85% NaCl and resuspended to 2%. Equal volumes (0.1 ml) of antiserum IgG, serially diluted in PBS, and sensitized erythrocytes were combined in wells of microtiter plates. Nonsensitized erythrocytes were used as controls. Plates were observed for agglutination after 3 hr of incubation at 37 C.

**Antigen preparations.** Clarified culture filtrate, used as an antigenic preparation in some experiments, was prepared by removing cells from broth culture by centrifugation and filtration through a 0.20-$\mu$m (pore size) filter. The filtrate was subsequently dialyzed against distilled water for 48 hr with frequent changes of water.

A phenol extract was obtained from acetone-dried cells by the phenol-water procedure for extraction of lipopolysaccharides from Gram-negative bacteria (27).

## RESULTS

**Antisera fractionation.** Antisera, fractionated on Sephacryl S300, separated into five components on the basis of absorbance at 280 nm (Fig. 1). Fractions with the highest titer corresponded with absorbance peak 4, which was identified as immunglobulin G (IgG) on the basis of reaction with specific anti-rabbit IgG antiserum (Miles Laboratories, Elkhart, IN 46515) and by reaction in commercial radial immunodiffusion plates (Miles) for measuring rabbit IgG. The remaining peaks were not identified, but peaks 2, 3, and 5 probably correspond with the IgM, $\alpha_2$-macroglobulin and albumin serum components, respectively (12). Peak 1 corresponded to elution of the column void volume.

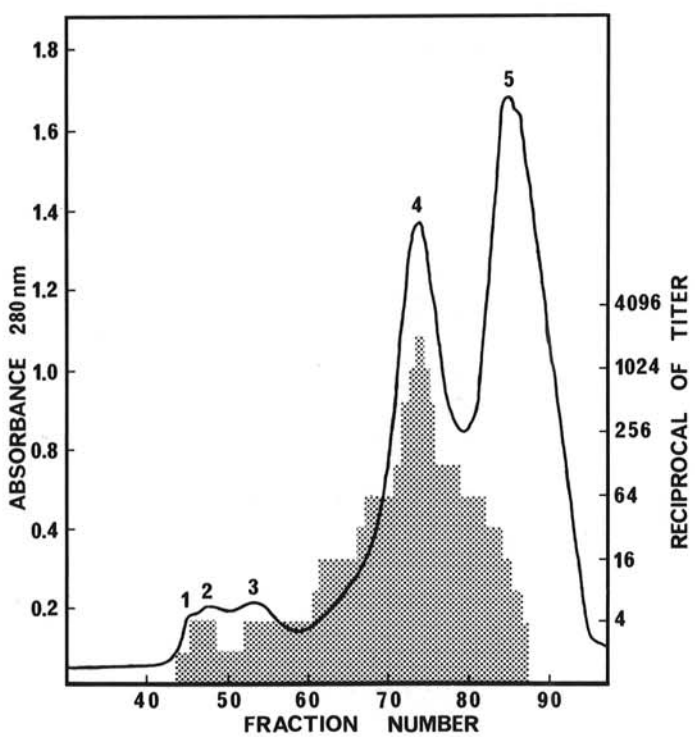


**Fig. 1.** Typical ultraviolet absorbance and immunofluorescence titer of fractions from *Corynebacterium sepedonicum* antisera fractionated on Sephacryl S300 column.

**Immunofluorescence cross-reactions.** The IgG fractions from all rabbit A bleedings were pooled and used to test cross-reactivity of strains of the *Corynebacterium* spp. listed in Table 1. All strains of *C. sepedonicum*, *C. insidiosum*, and *C. michiganense* were positive. In addition, a strain of *C. fascians* (ICPB CF17) and of *C. iranicum* (ICPB CI147) were weakly positive. All other strains were negative.

**Specific activity determinations.** Specific activity of the IgG fractions, measured against CS3, increased sharply during the second and third weeks after beginning immunization (Fig. 2). Subsequent intravenous and intramuscular injections did not increase titers beyond the initial response. The highest activity of rabbit A IgG was 2,470 units on day 59 and 718 units for rabbit B IgG on day 66. Activity of IgG from rabbit A against CI1 and strain A was almost equal to activity against CS3 in all bleedings. Activity against CM3 developed more slowly, but almost equalled the other activities by day 66 and exceeded activity against CS3 by day 158. Activity of IgG from rabbit B was almost the same against CI1, CM3, and strain A, but significantly lower than activity against CS3 until day 143 when activity approached that against CS3. By day 158, the activity against the heterologous strains exceeded that against CS3.

Specific activity of all IgG fractions from rabbit A were also determined against strains CS15, N53, and CM9, and from rabbit B against CI16 and CM8 with results very similar to that against strains of the same species in Fig. 2.

**Immunodiffusion.** In Ouchterlony double-diffusion tests, broth cultures of CI1, N, N53, CM3, and CM9 reacted with antiserum collected after day 24 from rabbits A and B (Fig. 3). Broth cultures of strain A produced very faint precipitin bands in some double-diffusion tests.

**Antigen preparations.** CS3 broth cultures prior to and after heat treatment at 121 C for 1 hr produced two precipitin bands in double-diffusion tests (Fig. 3). Clarified culture filtrates also gave two precipitin bands, but cells removed from broth cultures and suspended in the original volume of distilled water reacted poorly.

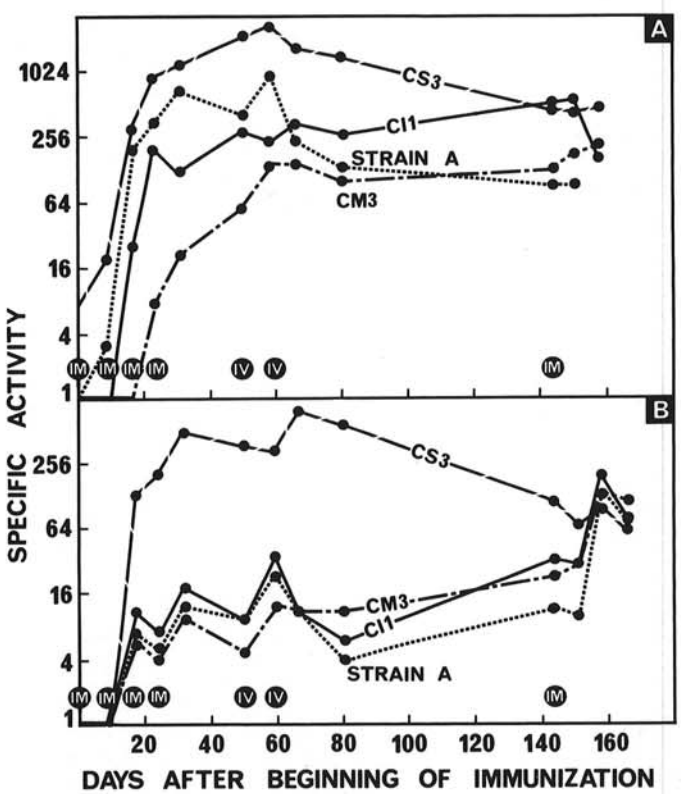


**Fig. 2.** Specific activity (immunofluorescence titer per milligram of protein) of IgG from two rabbits (**A** and **B**) measured against CS3 (*Corynebacterium sepedonicum*), CI1 (*C. insidiosum*), CM3 (*C. michiganense*), and strain A (an unidentified coryneform bacterium). IM and IV indicate time of intramuscular and intravenous injections, respectively.

However, the phenol extracts from whole washed cells reacted in double-diffusion tests showing the same two precipitin bands as the broth culture and, in addition, gave a third, barely visible, precipitin band between the other two.

The clarified culture filtrate activated sheep erythrocytes for indirect hemagglutination while the phenol extract did not (Table 2).

## DISCUSSION

In studies involving immunofluorescence techniques, antibody reaction is usually measured subjectively as fluorescence intensity, although photometric microscopy has been used for quantitative measurement (9). Dilution titer is commonly used with other serological techniques to determine quality of antisera and it was useful in this study with immunofluorescence to compare antibody reactions with homologous and heterologous bacterial species.

The specific activity data (Fig. 2) show that, as expected, antisera obtained soon after immunization begins have greater specificity than those taken at a later date. Furthermore, some *C. sepedonicum* antisera such as that from rabbit B can be diluted to minimize cross-reactions while for other antisera such as that from rabbit A, the difference between activity to homologous and heterologous strains is too small to eliminate cross-reactions by dilution. The greater activity against the heterologous strains compared to the homologous strains in antisera collected at the late dates reflect the results of Strobel and Rai (25), who found antiserum produced against a *C. michiganense* antigen to be more useful in serological tests for *C. sepedonicum* than antisera produced against a similarly prepared antigen from *C. sepedonicum*.

The greater cross-reactivity of the IgG from rabbit A compared to that from rabbit B may have been influenced by the individual immunological histories of the animals. A very low degree of reactivity in immunofluorescence tests against *C. sepedonicum* was detected in preimmune sera from rabbit A (Fig. 2). This may have been due to peptidoglycan or teichoic acid antibodies that are sometimes present in preimmune sera (7,11).

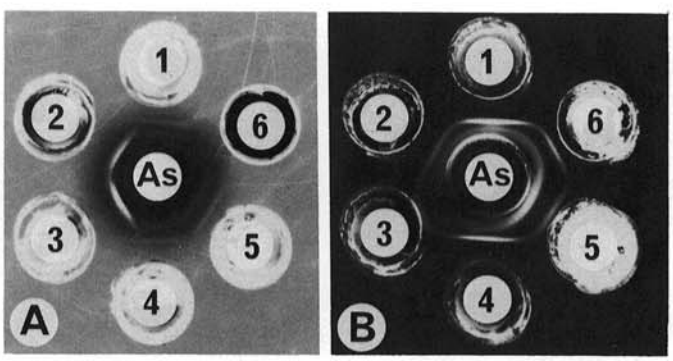


**Fig. 3.** Agar double diffusion against *Corynebacterium sepedonicum* antiserum. In series **A**, wells 1, 4, and 5 were charged with CS3 (*C. sepedonicum*) broth cultures, well 2 with CI1 (*C. insidiosum*), well 3 with CM3 (*C. michiganense*), and well 6 is empty. In series **B**, wells 1 and 4 were charged with CS3 broth, well 2 with CS3 culture filtrate, well 3 with washed CS3 cells, well 5 with CS3 phenol extract, and well 6 with heat-treated CS3 broth culture. Center As wells of both series were charged with antiserum from rabbit B. Series A was photographed after 5 hr at room temperature and series B was photographed after 20 hr at 5 C.

TABLE 2. Indirect hemagglutination of sheep erythrocytes activated with clarified culture filtrate and phenol extract from *Corynebacterium sepedonicum*

| | Reciprocal of indirect hemagglutination titer for the IgG fraction | | | |
|---|---|---|---|---|
| | Rabbit A | | Rabbit B | |
| | Day 31 | Day 158 | Day 31 | Day 158 |
| Culture filtrate | 128 | 8 | 16 | 32 |
| Phenol extract | 0 | 0 | 0 | 0 |

Reaction of *C. sepedonicum* in Ouchterlony double diffusion has been demonstrated previously by Slack et al (23) and precipitin band formation developed rapidly with antiserum from both rabbits used in this study when broth cultures were used. Antigenic activity of broth cultures was associated with the culture filtrate, but a similar antigen could be extracted from cells with hot phenol. Although Slack et al (23) were unable to detect serological cross-reactions with other bacteria in double diffusion, antisera produced during the current study cross-reacted with both *C. insidiosum* and *C. michiganense*. Cross-reactions, in double diffusion, of nonpathogenic bacteria isolated from healthy potato stems have also been found (5).

*C. sepedonicum* antigens have not yet been purified adequately to allow positive identification, but the phenol extraction procedure often is used to extract teichoic acids from Gram-positive bacteria (2,4,14). Furthermore, sensitization of unmodified erythrocytes with the culture filtrate suggests the presence of lipoteichoic acid since the procedure can be used semi-quantitatively for detection of lipoteichoic acid in culture supernatants of Gram-positive bacteria (10,17). The heat stability of the antigen also suggests a carbohydrate antigenic determinant.

The results indicate that certain *C. sepedonicum* antigens are probably similar to the surface antigens found on other Gram-positive bacteria. Certainly, serological cross-reactions with *C. michiganense* and *C. insidiosum* were demonstrated and have been reported by others (13,19,24,26). Weak cross-reactions with other plant pathogenic species including *C. flaccumfaciens* (13,24,26), *C. poinsettiae* (24), and *C. tritici* (15) have been noted and we detected cross-reactivity with a strain of *C. fascians* and *C. iranicum*. In at least one instance, when latex agglutination was used, cross-reactions may have been nonspecific since they were eliminated by addition of bovine serum albumin to the reaction mixture (24). However, the *C. insidiosum* and *C. michiganense* cross-reactions reported here appear to be due to specific antigens that are partially identical to *C. sepedonicum* antigens as indicated by spur formation of precipitin bands in double diffusion (Fig. 3). Serological similarity of *C. sepedonicum* to nonplant-pathogenic bacteria has not been studied in detail. One study did find *C. sepedonicum* antisera reacted with *C. diphtheriae* nucleoprotein extracts (15).

Cross-reaction of *C. sepedonicum* antisera with other bacteria limits the usefulness of immunofluorescence for detecting the presence of *C. sepedonicum* cells in potato tissue. Although the immunofluorescence procedure was useful in confirming the diagnosis of ring rot when a large number of cells were present (6), positive identification by immunofluorescence of low populations of ring rot bacteria in symptomless tissue was not possible (5). Antisera specificity could be enhanced by cross-absorption with known cross-reacting bacteria. However, cross-absorption with any one strain did not remove cross-reacting antibodies against all other strains and antisera absorbed with all known cross-reacting bacteria had significantly reduced titers to *C. sepedonicum* (5, and *unpublished*). Moreover, the possibility that yet other cross-reacting bacteria are associated with potato plants exists.

Further studies may identify common coryneform antigens as well as species specific antigenic determinants, which would allow highly specific detection of *C. sepedonicum* with sensitive serological techniques.

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