NONPATHOGENIC BACTERIA ASSOCIATED WITH POTATO STEMS CROSS-REACT WITH *CORYNEBACTERIUM SEPEDONICUM* ANTISERA IN IMMUNOFLUORESCENCE

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Abstract

Forty-three and 16% of stem smears from ostensibly healthy potato plants tested in 1980 and 1981, respectively, by immunofluorescence using *Corynebacterium sepedonicum* antisera, had fluorescing bacterial cells. Eight different bacteria that cross-reacted with *C. sepedonicum* antisera in immunofluorescence were isolated from stems. Four of these bacteria were Gram negative, three were Gram positive, and one was Gram variable. All bacteria differed from *C. sepedonicum* in morphological and biochemical characteristics except the Gram variable bacteria which were morphologically similar to *C. sepedonicum* at some growth stages. None of the cross-reacting bacteria was pathogenic on eggplant (*Solanum melongena* L. cv. Black Beauty). Three of the bacteria also formed precipitin bands in double diffusion with *C. sepedonicum* antiserum. Adsorption of antiserum with any one of the cross-reacting bacteria did not prevent immunofluorescence staining of all the isolated strains. Due to the cross-reactions, reliability of immunofluorescence for detection of latent bacterial ring rot infection was limited.

Resumen

Cuarenta y tres y 16% de los frotis de tallos provenientes de plantas sanas de papas probados en 1980 y 1981, respectivamente, por medio de immunofluorescencia usando antisuero de *Corynebacterium sepedonicum* presentaron células bacterianas fluorescentes. Ocho diferentes bacterias que reaccionaron con antisuero de *C. sepedonicum* en immunofluorescencia fueron aisladas de los tallos. Cuatro de estas bacterias fueron Gram positivo, tres fueron Gram negativo y una fue Gram variable. Todas estas bacterias fueron diferentes de *C. sepedonicum* en sus características morfológicas y bioquímicas, excepto la Gram variable cuyas características morfológicas fueron similares a las de *C. sepedonicum* el algunos de sus estados de desarrollo. Ninguna de estas bacterias fue patogénica en berenjena (*Solanum melongena* L. cv. Black Beauty). Tres de estas bacterias también formaron bandas de precipitación en doble difusión con antisuero de *C. sepedonicum*. La absorción de antisuero por cualquiera de estas bacterias

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no previno el teñido por immunofluorescencia de todos los strains aislados. Debido a estas reacciones cruzadas, la confiabilidad de la immunofluorescencia para detectar infección latente de pudrición anular fue limitada.

Introduction

As part of pathogen-free seed potato programs, potato stems or tubers are often routinely tested for the presence of *Corynebacterium sepedonicum* (Spieck. and Kotth.) Skapt. and Burkh., the causal agent of bacterial ring rot. In one procedure stem smears are observed for the presence of Gram positive bacteria as an indication of possible latent infection (2). Recently, we used the indirect fluorescent antibody staining (IFAS) technique in addition to the Gram stain procedure for detecting *C. sepedonicum*. Fluorescing bacterial cells were observed in some stem smears, some of which also contained Gram positive cells. But since serological cross-reactions of *C. sepedonicum* antisera with other bacteria have been reported (5, 8, 9), the IFAS positive bacteria in the stem smears may have been cross-reacting soil bacteria splashed onto the potato stems or non-pathogenic bacteria internally present in the potato stems (4). The objective of this study was to determine whether IFAS cross-reacting bacteria may be associated with healthy potato stems and interfere with the immunofluorescence detection of ring rot bacteria.

Materials and Methods

Detection and Isolation of Bacteria Cross-reacting with C. sepedonicum *Antiserum*

During 1980 and 1981 potato plants of several different varieties were grown in non-sterile field soil in greenhouses. About 10-12 weeks after planting, stems were cut 1 cm above the soil level and tested immediately or after storage at 4 C overnight. Plant sap (0.1-0.3 ml) was squeezed from the base of each stem with pliers into sterile test tubes. Sap from stems derived from the same seed piece were combined and tested as a composite sample. A loopful of sap from each sample was placed on each of two slides and the remainder of the sap stored at 4 C until needed. One slide of each sample was stained by the IFAS technique and the other slide was stained by Reed's modification of the Gram stain procedure (7).

If positive IFAS cells were found in a preparation, the corresponding sap sample was diluted in sterile, distilled water in 10-fold steps. Several dilutions were plated on YGM medium (5) and the plates were incubated 24-48 h at 28 C. Several colonies from each plate were selected on the basis of differences in colour, shape, size and texture, and stained by IFAS to determine whether they reacted with *C. sepedonicum* antisera. Cross-reacting bacteria were transferred to YGM slants, incubated for 48 h at 28 C

and stored at 4 C. Fresh cultures were routinely subcultured on YGM plates from these slants.

Immunofluorescence Staining

The IFAS technique as described previously (5) was used except that purified IgG (purification will be described elsewhere) rather than ammonium sulfate fractionated antisera, and anti-rabbit IgG antiserum conjugated with fluorescein (Litton Bionetics, Inc., Kensington, MD 20705) were used as reagents. IgG fractions from three different *C. sepedonicum* antisera (designated 1, 2 and 3) were used: antiserum 3 was obtained from Dr. S.A. Slack (University of Wisconsin-Madison). IgG from antiserum 1 diluted 1:10 was used for routine testing of stem smears in 1980 and IgG from antiserum 2 diluted 1:40 was used in 1981. IgG from all three sources was used to determine titers with cross-reacting bacteria.

Adsorption of C. sepedonicum *Antiserum*

Purified anti-*C. sepedonicum* IgG from antiserum 1 was adsorbed by suspending washed, pelleted (12,000 g for 10 min) 48-h-old cells in aliquots of IgG and incubating at 50 C for 1 h. Bacteria were removed by centrifugation and adsorption repeated once more. Complete removal of bacterial cells from IgG fractions was achieved by filtration through o.2 μ pore size filters. Separate aliquots of IgG were adsorbed with different bacteria and the adsorbed IgG used for IFAS.

Immunodiffusion Tests

Bacteria cross-reacting with *C. sepedonicum* antiserum in immunofluorescence staining were also tested for cross-reaction by Ouchterlony double diffusion. Agar plates were prepared with 15 ml of 0.8% Difco purified agar, 0.85% NaCl and 200 ppm sodium azide per 100 x 15 mm plastic petri dish. Six peripheral wells 5 mm in diameter were cut surrounding a center well of the same diameter; wells were 4 mm apart. Four-week-old broth cultures of test strains were placed in peripheral wells and the center well was charged with undiluted, unpurified antisera 1 or 2. Plates were incubated overnight at room temperature.

Characterization of Bacteria

Pathogenicity of IFAS cross-reacting bacteria was determined by inoculating eggplant (*Solanum melongena* L. cv. Black Beauty) using a technique modified from Lelliott and Sellar (6). Forty-eight-hour-old cultures were smeared with cotton swabs into a 1 cm longitudinal slit cut in stems between the two cotyledons of eggplants at the first leaf stage and sealed with petroleum jelly (vaseline). The inoculated eggplants were checked for symptoms at weekly intervals for eight weeks.

Standard microbiological procedures were used to perform the Hugh and Leifson test for oxidative/fermentative glucose metabolism, capsule staining, and nitratase test (3). Motility was observed on soft agar plates

containing 1% Difco-tryptone, 0.2% agar and 0.5% NaCl, 48 h after stab inoculation of cultures in the center of plates (1).

Results

All plants from which stems were used during both 1980 and 1981 were symptomless. Of 143 potato plants tested in 1980, 23% had Gram positive cells and 43% had IFAS positive cells in smears from sap expressed from the lower portion of the stems (Table 1). The potato plants tested in 1981 had fewer bacteria than plants tested in 1980. In 1981, 9% had Gram positive and 16% IFAS positive cells of 173 plants tested (Table 1). Although a

TABLE 1. — *Potato stems tested during 1980 and 1981 for presence of Gram-positive bacteria and bacteria cross-reacting with* Corynebacterium sepedonicum *antiserum in IFAS*[1] *tests.*

	Number of plants with positive bacteria					
	1980			1981		
Variety	No. plants tested	Gram-stain	IFAS	No. plants tested	Gram-stain	IFAS
Nooksack	6	2	3	11	0	0
Norchip	8	6	6	6	0	5
Norgold Russet	19	1	4	26	0	0
Norland	19	0	9	21	2	2
Red Pontiac	4	0	3	4	0	2
Russet Burbank	33	6	13	35	5	12
Warba	19	0	3	28	1	0
White Rose	11	9	5	15	1	0
other[2]	24	9	16	27	7	7

[1]Indirect fluorescent antibody staining

[2]Includes: Bintje, Butte, Early Epicure, Green Mountain, Hudson, Kennebec, Lemhi Russet, Red La Soda and Urgenta.

few of the preparations contained up to 20 IFAS positive cells/microscope field most preparations contained < 1 cell/field. About 33 and 10% of the preparations in 1980 and 1981, respectively, that contained IFAS positive bacteria also had Gram positive bacteria.

Eight different bacteria that reacted with *C. sepedonicum* antisera in IFAS tests were isolated from stem sap of potato varieties Russet Burbank, Red Pontiac, Green Mountain, Nooksack, and Butte tested in 1980 (Table 2). Four of the bacteria were Gram negative, three were Gram positive, and one was Gram variable. The Gram positive bacteria differed from *C. sepedonicum* by their rapid growth on YGM media (colonies > 1 mm in diameter after 48 h) and motility. They were also non-pathogenic on eggplant. Most of the isolated bacteria also differed from *C. sepedonicum* in cell morphology but the Gram variable bacterium looked very similar to *C.*

TABLE 2. — *Characteristics of bacteria isolated from ostensibly healthy potato stems compared to characteristics of* Corynebacterium sepedonicum.

Test	Bacterial strain								
	C. sepedonicum	A	B	C	D	E	F	G	H
Potato variety		Green Mountain	Russet Burbank	Nooksack	Red Pontiac	Red Pontiac	Russet Burbank	Butte	Russet Burbank
Cell shape	short rod	short rod	short rod	short rod	long rod	short rod	short rod	cocci	short rod
Cell size (μm)	0.4-0.6 x0.8-1.2	0.4-0.5 x1.0-1.2	0.7 x1.0-1.5	0.4 x1.0-1.5	0.8-1.0 x4.5-6.0	0.5 x1.2-1.5	0.5-0.6 x1.0-1.5	0.6-0.8	0.5-0.7 x1.2-1.5
Colony pigmentation	cream	cream	cream	yellow	cream	yellow	cream	bright yellow	pale orange
Gram stain	+	±	-	-	-	+	-	+	+
Glucose metabolism	0[1]	0	F	0	F	0	0	0	0
Capsule	-	-	+	-	-	-	-	-	+
Motile	-	+	+	+	+	+	+	+	-
Growth[2] slow	+	-	-	-	+	-	-	-	-
Nitrate reductase	-	-	+	+	-	-	-	+	-
Pathogenic[3]	+	-	-	-	-	-	-	-	-

[1]0 indicates oxidative metabolism, F indicates fermentative metabolism.
[2]Colonies on YGM medium < 1 mm diameter after 3 days.
[3]On eggplant (*Solanum melongena* L. cv. Black Beauty).

sepedonicum at certain growth stages. In old cultures, however, cells of this bacterium were pleomorphic ranging from coccoid to rod-like shapes with rudimentary branching. The cross-reacting bacteria were different from each other based on physiological and biochemical tests (Table 2).

Antisera titers with the cross-reacting bacteria were consistently lower than titers with *C. sepedonicum* cells (Table 3). Antisera 1 and 2 had the

TABLE 3. — *Immunofluorescence titers of IgG fractions of antisera produced against* Corynebacterium sepedonicum *and tested against* C. sepedonicum *and unidentified bacteria isolated from potato stems.*

Test strain	Reciprocal of titer				
	Conjugated anti-rabbit IgG alone	Preimmune	1	2	3
C. sepedonicum	0	0	256	256	32
A	0	0	128	32	4
B	0	0	8	4	0
C	0	0	32	16	16
E	0	0	32	8	4
F	0	0	2	32	1
G	0	0	64	0	0
H	0	0	4	8	0

same titer with the homologous bacteria but antiserum 1 had higher cross-reacting titers than antiserum 2 with all but two of the heterologous bacteria. Antiserum 3 had the lowest titer with the homologous bacteria and had correspondingly lower cross-reactivity. None of the bacteria reacted with pre-immune or conjugated anti-rabbit IgG antisera alone.

Adsorption of antiserum 1 with any one of the cross-reacting bacteria did not eliminate reaction with all the other strains, although adsorption with *C. sepedonicum* eliminated reactions with all isolated bacteria.

The Gram variable bacterium (A), one Gram positive bacterium (E), and two Gram negative bacteria (C, D; strain D was subsequently lost in culture) fluoresced brightly and uniformly in IFAS tests. Strain G fluoresced brightly with antiserum 1, but not all the cells in a preparation stained; it did not react with antisera 2 and 3. Strain F fluoresced brightly with antiserum 2 but staining was uneven with all antisera. The remaining strains B and H were capsulated and it appeared the stain was retained by the capsular polysaccharide.

Strains G and H produced precipitin bands with antisera 1 and 2 in double diffusion tests (Fig. 1). Strain A produced a very weak precipitin band in some tests. The spur reaction of the precipitin bands indicated partial identity of the unknown bacteria with *C. sepedonicum*.

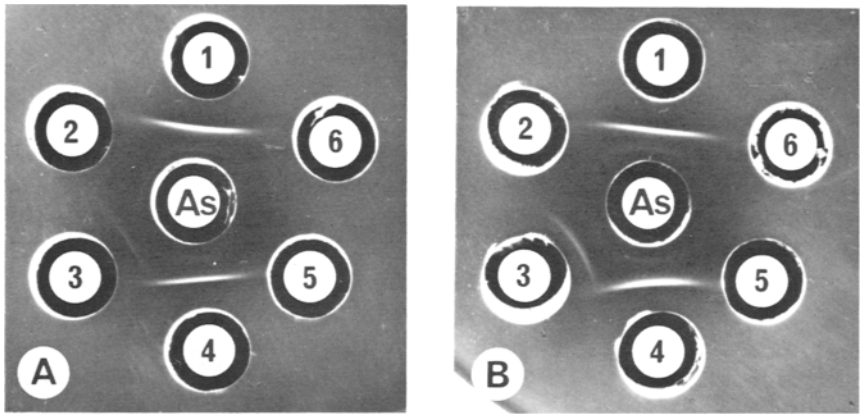

FIG. 1. Reaction in double diffusion of bacterial strains that cross-reacted in immunofluorescence with *C. sepedonicum* antiserum. Center wells of set A and B charged with unpurified antiserum 2; set A peripheral wells 1 and 4 charged with *C. sepedonicum* broth culture and wells 2, 3, 5 and 6 charged with broth cultures of strains A, H, E, and B respectively; set B peripheral wells 1 and 4 charged with *C. sepedonicum* broth culture and wells 2, 3, and 6 charged with broth cultures of strain F, C, and G, respectively; well 5 is empty.

Discussion

A high percentage of potato stem smears contained Gram positive and IFAS positive bacteria. Isolation of some cross-reacting bacteria from potato stems permitted direct comparison of the IFAS positive bacteria with *C. sepedonicum*. Isolated bacteria that fluoresced in the IFAS test differed from the ring rot pathogen in pathogenicity, cell and colony morphologies, and biochemical characteristics.

The serological relationship between *C. sepedonicum* and the nonpathogenic bacteria was confirmed for three of the isolated strains by reaction in double diffusion. Since adsorption with any one of the cross-reacting bacteria did not remove all cross-reacting antibodies, several different antigenic determinants were probably involved in the different cross-reactions. Adsorption with several different bacteria, therefore, would be necessary to render *C. sepedonicum* antiserum more specific. Staining of the cross-reacting bacteria from pure culture could be avoided by antiserum dilution but staining of *C. sepedonicum* in stem sap smears was too weak at dilutions beyond those used in this study.

The possible presence of bacteria that cross-react with *C. sepedonicum* antisera in association with healthy potato stems limits the usefulness of IFAS as a detection procedure for latent infections. The presence of some positive IFAS cells cannot be used as peremptory evidence for the presence of ring rot.

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