Immunofluorescent Stain Procedures for Detection and Identification of Erwinia carotovora var. atroseptica

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ABSTRACT

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A fluorescent antibody stain (FAS) was effective in the identification of isolates of *Erwinia carotovora* var. *atroseptica* (*Eca*) from different geographic areas. The antiserum for the FAS was prepared using bacterial cells (Wisconsin isolate - SR-8) fixed in 2% glutaraldehyde. All U.S. isolates of *Eca* from potato, which were confirmed by physiological tests, reacted with the FAS. Three isolates of *Eca* from potato (Scotland) and all the isolates of *Eca* from sunflower (Mexico), sugarbeets (U.S.), iris (England), and tomato (England) did not give a positive reaction. All other isolates of *Erwinia* spp. that were tested did not react with the FAS with the exception of one potato isolate of *E. carotovora* var. *carotovora* (*Ecc*) from The Netherlands. Fluorescing *Eca* cells were detected on prepared slides in mixed populations containing as few as 500 cells/ml mixed with 10^8 cells/ml of *Ecc*. Cells of *Eca* also were detected in soils to which as few as 10 cells/gm had been added and on potato leaves previously sprayed with bacterial suspensions. The FAS procedure also was effective in detecting *Eca* in tissues of infected and/or decaying potato tubers and in artificially contaminated adult fruit flies (*Drosophila melanogaster*).

Additional key words: blackleg, bacterial soft rot, *Erwinia chrysanthemi*, *E. aroideae*.

In contrast to its widespread application in medical microbiology, the technique of fluorescent antibody staining (FAS) for identification and detection of bacteria has been used infrequently in plant pathology and closely related fields. The potential effectiveness of this technique was well demonstrated by Schmidt and co-workers (24, 25) in studies of *Rhizobium japonicum* (Kirchner) Buchanan in soil, by Thomason et al. (27) with *Pseudomonas* (*Malleomyces*) *pseudomallei* (Whitmore) Haynes in mixed cultures and on leaf surfaces, by Paton (22) with *Pseudomonas syringae* van Hall in turnips, and by Auger and Shalla (2) for the detection of the Pierce's disease bacteria. The high specificity of FAS for particular isolates or strains used as the antigen source was observed for *Erwinia aroideae* (Townsend) Bergey et al. (12) and for *R. japonicum* (25).

Although progress has been made in the development of improved methods for detection and identification of soft-rot bacteria in potato tubers (*Solanum tuberosum* L.) (7, 8, 23), definitive identification of pectolytic *Erwinia* spp. obtained in isolations requires time-consuming biochemical and physiological tests to differentiate *E. carotovora* var. *carotovora* (Jones) Dye from *E. carotovora* var. *atroseptica* (van Hall) Dye (10).

In connection with a survey for blackleg bacteria in certified and commercial potato stocks, and projected ecological studies, there would be obvious advantages in the use of an FAS procedure. This investigation was initiated to determine whether specific FAS techniques could be adapted for detection and identification of *E. carotovora* var. *atroseptica* not only in pure culture and plant tissue, but also in soil and insects. A preliminary report on this research has been presented previously (1).

Erwinia carotovora var. *carotovora* and *E. carotovora* var. *atroseptica* will be referred to as *Ecc* and *Eca*, respectively, in the text and both varieties collectively will be referred to as *E. carotovora*.

MATERIALS AND METHODS

Preparation of antiserum.—Isolate SR-8 of *Eca* which was obtained in 1969 in Wisconsin from a potato stem (cultivar Early Gem) was grown on casamino acid-peptone-glucose agar (CPG) (11) at 24 C for 48 hr. Bacteria were washed three times in PBS [phosphate buffered saline = 0.01 M potassium phosphate buffer (pH 7.2) and 0.15 M NaCl] (14) and centrifuged at 12,000 *g* for 20 min. The washed bacteria were resuspended in PBS, dialyzed against 2% glutaraldehyde for 3 hr (26), and then dialyzed against PBS at 4 C for 20 hr with frequent changes of PBS. Intramuscular injections consisting of 1 ml of bacterial suspension (adjusted to $3\text{-}4 \times 10^9$ cells/ml with PBS) emulsified with 1 ml of Freund's incomplete adjuvant (Difco, Detroit, MI 48232) were made in the

TABLE 1. Agglutination and immunofluorescent reactions of different isolates of *Erwinia* spp.

Isolate number and other stock numbers	Location	Host	Agglutination[a]	FAS[b]
E. carotovora var. *atroseptica*:				
126[c], 128[c]	Florida	Potato	4	+
127[c]	Florida	Potato	3	+
150[c], 151[c], 152[c]	Minnesota	Potato	4	+
155[c]	New York	Potato	4	+
156[c]	New York	Potato	1	+
157[c], 158[c]	New York	Potato	2	+
159[c], 160[c], 161[c]	N. Dakota	Potato	4	+
55[c] (ICPB-EA 155; NCPPB 459)	Scotland	Potato	4	+
3[c], 8[c], 100[c], 103[c], 123c[c], 130[c], 133[c]	Wisconsin	Potato	4	+
123b[c], 135[c], 153[c]	Wisconsin	Potato	2	+
154[c]	Wisconsin	Potato	3	+
E. carotovora var. *carotovora*:				
SR 40 (ICPB-EC2)	California	Potato	0	−
41 (ICPB-EC3)	California	Celery	0	−
42 (ICPB-EC13)	California	Carrot	0	−
45 (ICPB-EC101)	California	Calla	3	−
46 (ICPB-EC102)	California	Carrot	2	−
47 (ICPB-EC105)	California	Potato	3	−
48 (ICPB-EC134)	California	Potato	2	−
49 (ICPB-EC144; ARK 4752)	California	Celery	4	−
50 (ICPB-EC146; KRAGHT 1)	California	— — —	0	−
51 (ICPB-EC153; ATCC 15359)	California	Mexican peppers	0	−
17	Florida	Tomato	0	−
52 (ICPB-EC169)	G. Britain	Broccoli	0	−
166[c]	Maine	Potato	0	−
165[c]	Minnesota	Potato	1	−
163[c]	New York	Potato	0	−
39	N. Carolina	— — —	0	−
129[c]	N. Dakota	Potato	1	−
162[c]	N. Dakota	Potato	0	−
53 (ICPB-EC208; ATCC 495)	Vermont	Carrot	0	−
13[c]	Wisconsin	Carrot	2	−
16[c], 123a[c], 131[c]	Wisconsin	Potato	0	−
136[c]	Wisconsin	Cabbage	0	−
164[c]	Wisconsin	Potato	1	−
E. chrysanthemi:				
SR 29, 30, 31 (ICPB-EC176), 32 (EC16), 33 (EC18)	New York	Chrysanthemum	0	−
E. chrysanthemi (corn stalk rot strain):				
SR 56 (EC209)	Egypt	Corn	1	−
120 (071-1230)	Hawaii	Corn	0	−
58 (C8)	N. Carolina	Corn	0	−
61 (W1-1), 80 (W3-20)	Wisconsin	Corn	1	−
E. aroideae:				
SR 36 (ICPB-EA144; Dowson 66)	England	Tobacco	3	−
37 (ICPB-EA149; Goto P8)	Japan	— — —	0	−
34	S. Carolina		0	−
38 (ICPB-EA13)	S. Carolina	Tobacco	0	−
E. carnegiana:				
SR 117 (E186)	Arizona	Giant cactus	0	−
E. amylovora:				
SR 122 (R_x395)	Missouri	Apple	1	−

[a]Relative amount of agglutination with serum diluted 1:20; 0 = no detectable agglutination; 1 = slight; 4 = strong positive.
[b]Fluorescent antibody stain reaction: + = distinct bright fluorescence; − = no fluorescence.
[c]Characterized on the basis of acid production from α-methyl glucoside, reducing sugars from sucrose, and absence of growth at 36 C (9).

hind leg of each of two New Zealand White test rabbits once a week for 4 wk. One wk after the fourth injection, rabbits were test-bled and the agglutination titer was determined by the microagglutinin test (3). Dilution series of the nonfractionated serum and of suspensions of SR-8 cells (10^7-10^8 cells/ml) were prepared in PBS for the titration. Injections were continued until the agglutination titer was greater than 1:2,560.

Fractionation of antiserum.—The antiserum was fractionated by the dropwise addition of saturated ammonium sulfate solution (6, 16). For agglutination and Ouchterlony test reactions, the final ammonium sulfate concentration was 50% (v/v); for FAS, the final ammonium sulfate concentration was 40% (v/v). After continuous stirring in the cold for 4 hr, the mixture was centrifuged; the precipitate was dissolved in PBS equal to the original volume and re-precipitated twice more. The final solution was dialyzed against several changes of PBS in the cold for 48 hr.

Conjugation of antiserum.—Conjugation of the globulin fraction of the antiserum with fluorescein-isothiocyanate (FITC) (Calbiochem, La Jolla, CA 92037) was completed with the following modifications of the simplified method I of Lewis et al. (16): (i) conjugation was continued for at least 6 hr at pH 9.5 rather than pH 10.5; (ii) 0.1 M carbonate-bicarbonate buffer (pH 9.5) was used to adjust the antiserum fraction to 1% protein and to dissolve the FITC; and (iii) after the conjugate was recovered from the Sephadex column, the pH was decreased to 7.2 by dialysis against PBS. The conjugate then was filter-sterilized and stored in serum bottles in the dark at 4 C. Addition of 1 mg/ml merthiolate (E. Lilly Co., Indianapolis, IN 46206) was found to be necessary for prolonged storage.

Staining procedures.—Cultures used to evaluate the specificity of the FAS were grown on CPG or King's B Medium (13). Pectolytic soil organisms were isolated using the crystal violet pectate (CVP) medium of Cuppels and Kelman (7). Slide preparations of these isolates were air-dried, heat-fixed, and stained with FAS in a moist chamber in the dark for 30 min (16). After rinsing with PBS for 10 min, the slides were rinsed quickly with deionized water and mounted with phosphate buffered glycerol [glycerol: 0.02 M phosphate buffer (pH 7.6) (9:1, v/v)].

Smears from decayed potato tissue were made by suspending tissue from the edge of the decayed area in 0.1 ml of sterile double-distilled water and smearing a loopful of the suspension on a slide. The slides then were heat-fixed and stained in the same manner as pure cultures.

To detect the bacteria on leaves, sterile cotton swabs moistened with sterile double-distilled water were used to swab the leaf surfaces; swabs then were rolled on surfaces of microscope slides (27).

Smears from adult fruit flies (*Drosophila melanogaster* Meigen) were prepared by grinding a single insect in 0.1 ml sterile double-distilled water and smearing a loopful of the resulting suspension on a slide.

To sample soils, 10 g of field soil were mixed and shaken with 100 ml of sterile double-distilled water for 45 min (120 rotations/min at 24 C). A flocculating agent [a mixture containing crystalline $Ca(OH)_2$ and $MgCO_3$ (2:5, v/v)] was then added (0.7 g) to the soil suspension and the mixture was shaken vigorously (5). The suspension was allowed to settle for approximately 1 hr; 40 ml of the clear

TABLE 2. Fluorescent antibody stain (FAS) reactions of isolates of *Erwinia carotovora* var. *atroseptica* (*Eca*) and *E. carotovora* var. *carotovora* (*Ecc*) from different geographic areas and hosts

Geographic location	Source of isolates	FAS test summary[a]	
		Eca	*Ecc*
USA:			
Arizona	Potato	3/3	0/4
California	Potato	...	0/2
	Yellow calla	...	0/1
	Sugarbeet	0/2	...
Colorado	Potato	18/18	0/13
	Insect	3/8	0/7
Connecticut	Potato	...	0/1
Florida	Potato	5/5	0/1
	Pepper	...	0/4
	Lettuce	...	0/1
	Carrot	...	0/6
	Chrysanthemum	...	0/1
	Caladium	...	0/1
Maine	Potato	1/1	0/1
Minnesota	Potato	7/7	0/1
New York	Potato	12/12	0/14
	Iris	...	0/1
North Dakota	Potato	3/3	...
Washington	Sugarbeet	0/4	...
Wisconsin	Potato	4/4	0/6
	Carrot	...	0/2
	Soil	1/1	0/6
Other countries:			
Canada	Potato	...	0/1
England/Scotland	Potato	9/13	...
	Tomato	0/1	...
	Iris	0/1	...
	Insects	2/2	...
Germany	Potato	...	0/1
Holland	Potato	1/1	1/1
Israel	Corn	...	0/1
Italy	Potato	2/2	...
Mexico	Sunflower	0/4	0/1
Peru	Potato	1/1	0/8

[a]Number of isolates reacting with the fluorescent antibody stain specific for *Eca* / no. of isolates tested.

TABLE 3. Number of fluorescent cells in mixtures of *Erwinia carotovora* var. *atroseptica* (*Eca*) with 10^8 cells/ml of *E. carotovora* var. *carotovora* on a slide smear stained with fluorescent antibody specific for *Eca*

Population of *Eca* (cells/ml)	Fluorescing cells (no.)
5×10^7	25.0[a]
5×10^6	7.0[a]
5×10^5	1.0[a] 24[b]
5×10^4	4[b]
5×10^3	0.3[b]
5×10^2	0.2[b]
5×10	0[b]
0	0[b]

[a]Average no. per field based on 10 fields (1 field $\simeq$ 0.13 mm^2).
[b]Average no. per scan based on six scans (area of scan $\simeq$ 6.08 mm^2).

supernatant fluid then was centrifuged at 12,000 *g* for 20 min and the pellet was resuspended in 1 ml of sterile double-distilled water. A drop of this suspension was placed on a slide.

Slide smears from leaves, fruit flies, and soils were air-dried, heat-fixed, and counterstained with 2% hydrolyzed gelatin conjugated with Rhodamine B-Isothiocyanate (Nutritional Biochemicals Corp., Cleveland, OH 44101) (4). The gelatin film was dried at 60 C and cooled to room temperature, then stained with the FAS in the same manner as the pure cultures.

Optical equipment.—All slides were examined using a Zeiss Universal photomicroscope with a high-pressure mercury ultraviolet (UV) lamp (HBO-100 W/2). A BG 12 exciter filter and a barrier filter 50 (transmission range above 500 nm) were used under epi-illumination at a magnification of × 625. Photographs were taken with Kodak Tri-X film under the same illumination, but the magnification was × 500.

Ouchterlony plate procedure.—Plates were prepared with 0.7% washed Ionagar No. 2 (Oxoid; Consolidated Laboratories, Inc., Chicago Heights, IL 60411) in 0.02 M potassium phosphate buffered physiological saline and 0.02% sodium azide (3, 21). A template was used to cut seven wells of equal diameters (5 mm) with a distance of 6 mm between wells. Antigen was prepared by grinding 2 ml of bacterial suspension (5×10^8 cells/ml) with ~1 cc of glass beads (0.17-0.18 mm diameter) at top speed on a Vortex-Genie mixer (Sci. Products, Mc Gaw Park, IL 60201) for 2 min. The antiserum of *Eca* (SR-8) was diluted 1:4 and placed in the center well. Disrupted cells of *Eca* (SR-8) were placed in alternate outer wells and the disrupted cells of other isolates were placed in the remaining three alternate outer wells. Plates were allowed to incubate at a temperature of approximately 20 C in a moist chamber for 2-3 days before readings were taken. The following isolates were tested: 16 *Eca* cultures, including 13 from potato and three from other hosts; 22 *Ecc* cultures; 19 cultures of other *Erwinia* spp. and 12 cultures of bacteria from other genera.

RESULTS

Specificity of antiserum.—Specificity of antiserum initially was evaluated by means of agglutination and fluorescent antibody stain reactions (Table 1). Agglutination test reactions were determined at a 1:20 dilution of antiserum. *Erwinia* spp. cultures tested included isolates from diverse geographic areas and different hosts (Table 1). In the agglutination tests, 15% of 66 cultures of *Erwinia* spp. showed cross-reactions, whereas in the fluorescent antibody tests no cross-reactions were observed.

These initial observations indicated that the FAS was highly specific for *Eca* and were confirmed with other isolates (Table 2). All 81 isolates of *Eca* obtained from potato, soil, or insects reacted with the FAS except three potato isolates from Scotland (G218, G221, and G222, provided by C. Quinn and D. C. Graham), one isolate from England [NCPPB 138 (Dowson) provided by R. Lelliott], and five isolates from insects from Colorado (provided by M. Harrison). The isolates designated as *Eca* obtained from sunflower (*Helianthus annuus* L.) in Mexico (provided by L. Fucikovsky), from sugar beet (*Beta vulgaris* L.) in Washington and California (provided by M. Harrison), and from iris (*Iris xiphioides* Ehrh.) and tomato (*Lycopersicon esculentum* Mill.) in Scotland (provided by C. Quinn) did not react with the FAS. The potato isolate of *Eca* obtained from Peru (provided by E. French) reacted with the FAS.

Of the 86 *Ecc* cultures tested, only one isolate from potato (provided by H. Maas-Geesteranus 1.39 - F strain) reacted with the FAS; all other isolates did not (Tables 1, 2).

No reaction with the FAS was noted with 16 isolates of other *Erwinia* spp. as well as with the following 60 isolates: one isolate each of *Pseudomonas coronafaciens*, *P. glycinea*, *P. phaseolicola*, and *P. tabaci*; *Bacillus cereus* var. *mycoides*, *B. polymyxa*, *B. pumilus*; *Proteus mirabilis*; *Enterobacter cloaceae*; *Staphylococcus aureus* *Serratia marcescens*, and *S. lactis*; two each of *P. fluorescens* and *Escherichia coli*; three of *Rhizobium japonicum*; five of *Clostridium* spp.; six of *P. marginalis*, and 31 other miscellaneous cultures of bacteria obtained in isolations from soil and decaying potato tubers.

A random sample of *Erwinia* isolates was tested against the *Eca* (SR-8) antiserum by means of a modified Ouchterlony plate method (3, 21). All of the 13 *Eca* cultures isolated from potatoes gave two identical precipitin lines with the *Eca* (SR-8) antiserum. The *Eca* isolates from tomato, iris, and sunflower gave no reaction with the antiserum; 22 *Ecc* cultures also did not react. The one *Ecc* culture (Maas-Geesteranus 1.39 - F strain) that was FAS positive gave three precipitin lines, including one line of partial identity; nevertheless, it was still easily differentiated from the *Eca* precipitin pattern. All 19 isolates of other *Erwinia* spp. as well as 12 isolates of other genera gave either no reaction or a nonidentical reaction with the antiserum.

Sensitivity of the FAS for detection of *Eca* in a mixed population was evaluated by preparing slides with suspensions of *Eca* mixed with high populations of *Ecc* (Table 3). With this procedure, *Eca* was detected when the sample contained as few as 500 cells/ml mixed with 10^8 cells of *Ecc* per milliliter.

Detection in tubers.—The FAS technique also was evaluated for detection of *Eca* in potato tubers by comparing its efficiency with that of the CVP plating method. In connection with a survey for blackleg bacteria in certified and commercial potato stocks from 76 farms in Wisconsin (Allan and Kelman, *unpublished*), over 1,100 tubers were wounded, wrapped in wet paper towels and one layer of polyvinylidene film (Saran Wrap, Dow Chemical Co., Indianapolis, IN 46204), and incubated for 5 days at 20 C (8). Then tissue segments were taken from the edges of decayed areas and suspended in 0.1 ml of sterile double-distilled water. One loopful of the suspension was streaked on CVP and a second loopful was smeared on a slide and stained with FAS. Fluorescing cells could be detected readily in these preparations from rotted potato tissue. Under phase-contrast (Fig. 1-A), the field shows the high number of bacteria present, including large, dark, clostridia-like cells. Under UV epi-illumination (Fig. 1-B), the background is efficiently blocked out and only the fluorescing bacteria are visible.

Identification of *E. carotovora*-type colonies growing on the CVP as *Eca* was based initially on three characteristics: (i) acid production from α-methyl

glucoside, (ii) formation of reducing sugars from sucrose, and (iii) absence of or slight growth at 36 C (10). Subsequently, cultures from *E. carotovora*-type colonies were confirmed as *Eca* mainly by the FAS procedure.

In 85% of the samples, results obtained with CVP platings were in agreement with those obtained with the FAS procedure (Table 4). In those instances when the two tests did not agree, the FAS procedure was more sensitive than CVP platings. In 14% of the samples, *Eca* would not have been detected if isolations had been made only on CVP. In one instance when *Eca* was detected with FAS and not on CVP, a smear of bacteria from heavy growth on the CVP plate was stained. Small, brightly fluorescing bacteria were present, but were less than 1% of the total bacterial population visible by phase-contrast optics. Thus, although *Eca* may be present in a culture, it may not always be able to compete successfully against high numbers of other bacteria on the CVP medium.

Detection of potato leaves.—To evaluate the FAS procedure for detection of *Eca* on potato leaves, detached leaves were sprayed with five dilutions of *Eca* suspended in sterile, double-distilled water (4×10^1 to 4×10^5 cells/cm^2 leaf surface), allowed to dry in the open for 2 hr, and held in sterile petri dishes overnight (27). The leaves then were sampled with moist cotton swabs. Slide smears were made with the swabs and stained with the FAS; plates of CVP also were streaked with the cotton swabs. With the use of both the CVP and FAS procedures, the bacteria could be detected at 4×10^4 cells/cm^2 leaf surface. At 4×10^3 cells/cm^2, no *E. carotovora* type colonies were detected on the CVP plates, but the low numbers of *Eca* could still be detected with the FAS. *Eca* was not detected with either method when cell numbers were less than 4×10^3 cells/cm^2.

In an additional test, potted potato plants (cultivars Russet Burbank and *Solanum phureja*) were sprayed with a water suspension of *Eca* (about 10^7 cells/ml) and maintained under growth-room conditions at 28 C. After 3 wk, *Eca* was still detected on the leaves with the FAS procedure. Numbers of viable *Eca* cells started to decline after 4 days; they were still detected using CVP after 9 days on *Solanum phureja* and after 17 days on Russet Burbank leaves.

Detection in insects and in soil.—To test the efficiency of FAS for detection of *Eca* in insects, adult fruit flies were placed in a quart jar in which *Eca* had been allowed to grow for 2 days on CPG. After 4 hr, the flies were transferred to a jar containing standard *Drosophila* growth medium. The flies were sampled immediately following exposure, and daily for 4 days thereafter. High

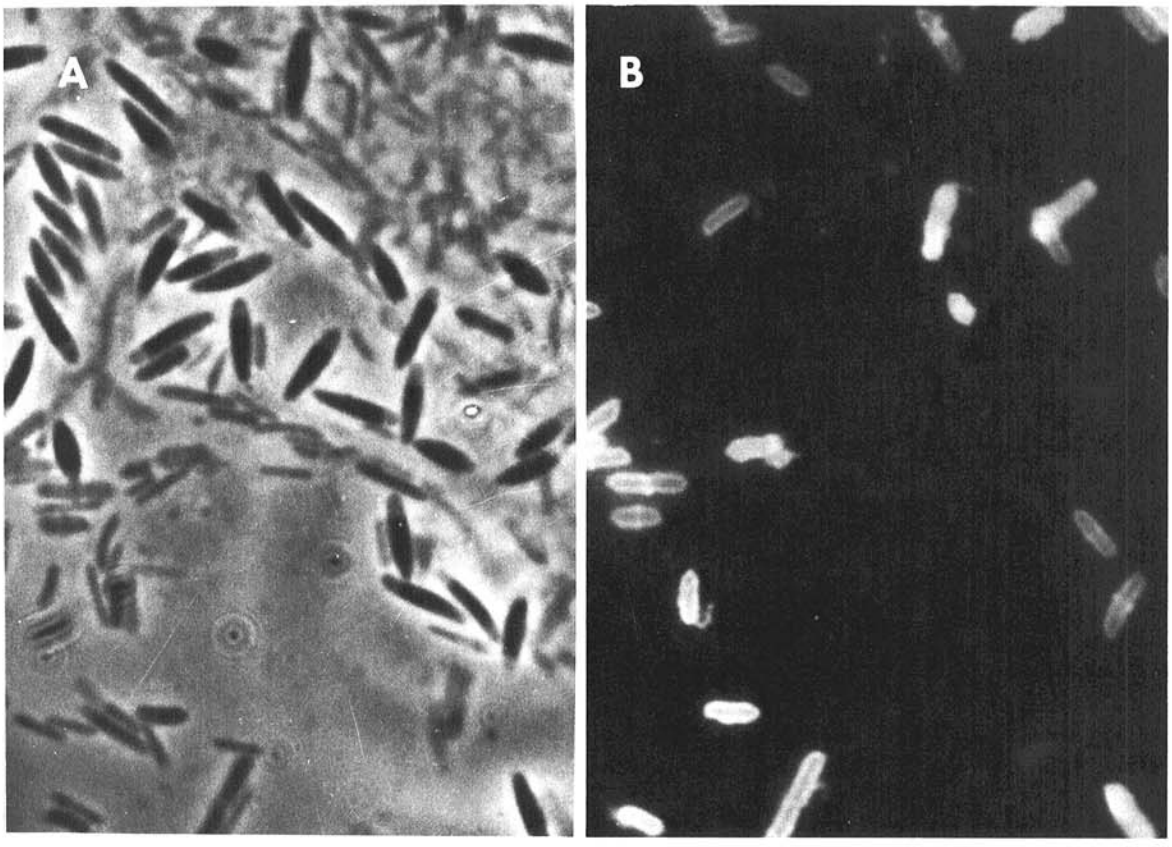

Fig. 1-(A, B). A) Phase-contrast photomicrograph of a slide coated with a mixed suspension of bacterial cells from rotted potato tissue showing large cells of spore-forming bacteria, those of other bacteria and of *Erwinia carotovora* var. *atroseptica* (*Eca*). **B)** Photomicrograph of same microscopic field shown in A. Cells were stained with the fluorescent antibody specific for *Eca* and photographed with ultraviolet (UV) epi-illumination. Only cells of *Eca* are visible under UV illumination. (× 500)

populations of *Eca* were detected in the insects at the start of the test and on the 2nd day by means of the FAS procedure. Populations of *Eca* in the insects declined after the 3rd day, but still could be detected on the 4th day.

Seedcorn maggot flies (*Hylemia platura* Meigen) collected in three different potato fields in which a high percentage of blackleg plants were present were assayed for the presence of *Eca* using the FAS procedure. Positive readings were obtained in 15 of 29 smears prepared from individual seedcorn maggot flies. In subsequent tests with collections of seedcorn maggot flies, positive tests with the FAS were correlated with isolations of *Eca* on CVP and identification by biochemical tests.

To evaluate the efficiency of FAS for detection of *Eca* in soils, cells were suspended at seven different concentrations (5×10^2 to 5×10^8) in sterile double-distilled water and 0.2 ml of each concentration were added to 10 g of clay-loam soil (cabbage field plot). Samples were mixed, allowed to stand at room temperature overnight, and treated as described under Materials and Methods and stained with FAS. Before the flocculant was added, 0.1 ml soil suspension was plated on CVP to count viable cells.

Although populations of *Eca* decreased markedly during the 1-day incubation period, it was possible on CVP to detect 40 viable cells/g in the samples to which 10^4 cells/g were added initially. On CVP, this soil had a background population of 10^6 cells/g of common soil-inhabiting bacteria. In those soil samples to which less than 10^4 cells/g had been added, no viable cells were recovered on CVP after the 1-day incubation period. In contrast, fluorescing bacteria were detected by use of FAS in the complete range of soil samples (10^7 cells/gm to 10 cells/gm). When less than 10^5 cells/g were added, the fluorescing bacteria were difficult to detect, however.

DISCUSSION

The fluorescent antibody stain had a high degree of specificity for *Eca*. This was based on tests with over 300 isolates of *Eca* and closely related species (Tables 1, 2) and other genera. A high degree of correlation also was observed between the results obtained with the FAS procedure and the Ouchterlony plate technique in which the same antiserum was tested.

In a similar study, the FAS procedure was evaluated by Kikumoto and Sakamoto (12) and the procedure was considered to be of limited value because of the wide diversity of strains present naturally in the soils that they sampled. In contrast with their results, our studies showed that isolates of *Eca* from potato obtained from diverse geographic areas reacted with the FAS with a high degree of uniformity.

The high specificity of the FAS procedure is an improvement over the slide agglutination tests which have been evaluated previously for identification and detection of *Eca* in potato tubers (9, 20). One of the techniques used in this study, which differed from standard procedures, was the use of glutaraldehyde in the fixation of bacterial cells prior to injection. Formaldehyde fixation had been used previously (19), but most serological studies have used heat-killed or sonicated cells for injection.

With few exceptions, all isolates from potato which were identified biochemically as *Eca* reacted with the FAS prepared in this study. The three potato isolates from Scotland which did not react with our antiserum are uncommon serotypes. They gave typical reactions in biochemical and pathogenicity tests, but did not react with any of the four *Eca* antisera prepared in Quinn's laboratory (Quinn, *personal communication*). The isolate sent to us by Lelliott (Dowson 138) also was an atypical, weakly pathogenic strain of *Eca* (Lelliott, *personal communication*). All the *Eca* isolates from hosts other than potato (four from sunflower, six from sugar beet, one from tomato, and one from iris) differed serologically from the potato isolates. None reacted with the FAS, although all were typical *Eca* in physiological tests. The five cultures isolated from insects by Harrison (Table 2) might also be nonpotato serotypes since they had been obtained from flies collected over fields planted to other crops (Harrison, *personal communication*). The three *Eca* cultures isolated from insects which had been collected over potato fields reacted with the FAS.

The isolate of *Eca* from Peru came from a potato plant grown in the locality of Comas near Huancayo in the central Andean Valley of the Montaro River where potatoes have been grown throughout recorded history. This area may not have been contaminated with potatoes imported from Europe (French, *personal communication*).

All the isolates of other *Erwinia* spp. and other genera did not react with the FAS with one exception. The *Ecc* isolate from The Netherlands (1.39 - F strain) was more similar serologically to *Eca* than all the other *Ecc* tested (28). The Ouchterlony plate procedure showed that there is one common component between this isolate and the *Eca* isolate SR-8.

In addition to rapid and reliable identification of cultures, the FAS procedure enables one to determine whether individual cultures of *Eca* are pure and, if not, to

TABLE 4. Relative efficiency of the fluorescent antibody stain (FAS) procedure in comparison to crystal violet pectate (CVP) plating procedures for detection of *Erwinia carotovora* var. *atroseptica* (*Eca*)

Comparisons	Tubers[a] sampled (no.)	Fraction of tubers with *Eca* detected (%)
Eca detected with CVP and FAS	32	3
Eca detected with CVP, but not FAS	13	1
Eca detected with FAS, but not CVP	164	14
Eca not detected with CVP or FAS	977	82

[a]Total number of tubers sampled = 1,186.

determine the relative numbers of *Eca* in the mixed population.

Two methods for detection of *Eca* in potato tubers were developed. In the isolations made on the CVP medium from rotted potato tissue, it was possible to select typical colonies of *E. carotovora* (7) and identify them with FAS. This eliminated the delay associated with isolating and restreaking individual colonies for purification, and completing routine biochemical tests for identification. Detection of bacteria by direct staining of decayed potato tissue also was possible and more sensitive than direct isolation on CVP. Although other bacteria and plant material were present, the brightly fluorescing *Eca* cells were detected easily.

These two methods were used effectively in an extensive survey of 76 farms to determine the prevalence of blackleg bacteria in certified and commercial potato stocks in Wisconsin. A survey of this kind involving examination of over 1,000 potatoes would have been very time-consuming if biochemical tests of pure cultures had been required to differentiate *Eca* from *Ecc*.

It was thought that the FAS procedure could be used to detect *Eca* directly in tuber lenticels. However, this procedure has certain limitations because of the low population of bacterial cells. Anaerobic incubation procedures following wounding of lenticels by wrapping tubers with Saran Wrap (8) or incubation in a mist chamber (17) provide alternative approaches. Under near-anaerobic conditions, *E. carotovora* cell populations increase and this facilitates the use of the FAS procedure as a stain for direct examination of the rotted tissue.

An enrichment technique for detection of low populations of *Erwinia* in soil has been used effectively by Meneley and Stanghellini (18). This procedure is an improvement over methods which involve direct baiting techniques (15) or isolations on selective media. However, in ecological studies, the FAS procedure may enable quantitative detection of *Eca* when it is present in relatively low numbers (10-100 cells/gm soil).

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