Use of Direct and Indirect Immunofluorescence Tests for Identification of Xanthomonas campestris

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ABSTRACT

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A comparison was made between direct and indirect immunofluorescence (IF) for rapid identification of *Xanthomonas campestris*. Eighteen strains of *X. campestris*, five of *X. vesicatoria*, nine of *Xanthomonas* spp., five of nonidentified yellow-pigmented bacteria, one of *Escherichia coli*, and one of *Pseudomonas syringae* were tested. For direct IF, cells were stained with fluorescein isothiocyanate (FITC)-conjugated γ globulin to bacterial ribosomes. For indirect IF, bacterial cells were treated with antiserum or γ globulin to ribosomes and stained with FITC-conjugated antirabbit globulin. The stained cells were observed by fluorescence microscopy. All strains of *X. campestris* tested by indirect IF were positive, and only *X. vesicatoria* cross-reacted with *X. campestris*. It is concluded that indirect IF using antiserum to ribosomes is a useful method for quick identification of *X. campestris*.

Xanthomonas campestris, the causal agent of black rot of crucifers, is a destructive, seed-borne pathogen (14, 15). With improved methods of detecting *X. campestris* in seeds (2, 10), a more rapid method of confirming the pathogen's identity is needed. Time-consuming pathogenicity tests currently are the only practical methods available. In such tests it takes 14-21 days for symptoms to appear in inoculated seedlings. Immunofluorescence (IF) is a simple and rapid test commonly used in clinical diagnosis of the agents of human diseases (3). Although IF tests have been suggested for identifying several plant pathogenic bacteria (1, 7, 8, 13), *X. campestris* has not been tested nor have the direct and indirect tests been compared using a plant pathogenic bacterium. The purpose of this investigation was to compare direct and indirect IF for identification of *X. campestris* isolated from crucifer seeds.

MATERIALS AND METHODS

Bacterial strains.—A listing of the strains used in this work and their sources is given in Table 1.

Antisera and γ globulin.—Antisera were produced by injecting New Zealand white rabbits with 70S ribosome preparations from *X. campestris* B-1 and *X. vesicatoria* B-215 as described elsewhere (9). The γ globulin fractions were prepared by DEAE Sephadex A-50 column chromatography (4).

Preparation of cells for staining.—Smears were made from a suspension in 0.85% NaCl (saline) of cells from a 24-hr yeast extract-dextrose-$CaCO_3$ (16) slant culture. The smears were made on multiwell slides (Cel-Line Associates, Inc., Minotola, NJ 08341) with a 0.001-ml loop. After drying they were flooded with Kirkpatrick's fixative (60% ethanol, 30% chloroform, and 10% formalin), placed in a humidity chamber for 3 min, rinsed with fixative, drained, and allowed to air-dry before staining.

Direct immunofluorescence.—The γ globulin was conjugated with fluorescein isothiocyanate (FITC), 99% pure (Biological Supply Co., Melbourn, FL 32901) at pH 9.5 in phosphate buffer and the molecular relationship between FITC and globulin protein (F/P ratio) was determined with fluorescein diacetate as a reference standard (6). Bacteria were stained by placing a drop of conjugate on a smear and incubating the slide in a humidity chamber in the dark for 30 min. The slides were rinsed in saline and then in 0.1 M phosphate buffered saline (PBS), pH 9.0, for 10 min. They then were rinsed in distilled water and mounted in 0.05 M carbonate-buffered (pH 9.0) glycerin (12).

Indirect immunofluorescence.—Bacteria were treated with antiserum or unlabelled γ globulin to 70S ribosomes and stained with a commercial fluorescent antirabbit globulin (Gibco Diagnostics, Grand Island, NY 14072) as described by Goldman (5), and mounted as above. A block test (5) was used to determine the optimum dilution of antirabbit globulin.

Fluorescence microscopy.—Stained preparations were examined under an ×40 objective fitted to a Zeiss Orthophot microscope with an oil immersion condenser. A Zeiss HBO-200 mercury vapor lamp (direct current power supply) was used as the exciting light source. A BG-12 interference filter was used for primary filtration and a No. 47 filter as the barrier filter. Staining titers were determined by taking the highest dilution of anitserum or γ globulin that gave a clear fluorescent stain (cells clearly visible). A titer of 0 indicated no fluorescence at the lowest dilution (1:4).

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TABLE 1. Comparison between direct and indirect fluorescent-antibody staining titers of *Xanthomonas campestris* and *X. vesicatoria* and several other bacteria[a]

Antigen	Strain	Host and habitat	Source[c]	Titers with antiserum or γ globulin			
				X. campestris B-1		*X. vesicatoria* B-215	
				Direct	Indirect[b]	Direct	Indirect[b]
X. campestris	B-45	Broccoli, seed (Japan)	(1)	8	64	0	4
	B-2	Kohlrabi, plant (Ga.)	(1)	ND	64	ND	ND
	B-1 (BBS)	Brussels sprouts, plant (Calif.)	(2)	32	32	0	0
	B-30 (K-2)	Unknown (Germany)	(3)	8	32	0	0
	B-25	Broccoli, seed (Japan)	(1)	4	32	4	0
	B-22	Cabbage, plant (Ga.)	(1)	4	32	4	0
	B-33(Q-19)	Brussels sprouts, plant (N. Zealand)	(4)	4	32	0	0
	B-26	Cabbage, plant (Ga.)	(1)	4	32	0	0
	B-36	Cauliflower, seed (Japan)	(1)	4	32	0	0
	B-11	Cabbage, plant (Ga.)	(1)	0	32	0	0
	B-47	Broccoli, seed (Japan)	(1)	ND	32	ND	ND
	B-44	Cauliflower, seed (Calif.)	(1)	8	16	0	0
	B-15	Cabbage, soil (Fla.)	(1)	8	8	0	4
	B-51	Cabbage, seed (Japan)	(1)	8	ND	0	0
	B-13	Cabbage, plant (Fla.)	(1)	4	8	0	16
	B-24	Broccoli, plant (Ore.)	(5)	4	8	0	0
	B-32 (Q-12)	Cabbage, plant (N. Zealand)	(4)	4	8	0	0
	B-52	Cabbage, seed (New Jersey)	(1)	4	ND	0	4
X. vesicatoria	B-218 (555-81)	Tomato (Del.)	(6)	8	8	4	64
	B-202	Pepper (Ore.)	(5)	0	8	0	64
	B-220 (X-5)	Tomato (N. Zealand)	(4)	4	8	4	64

	B-228 (ENA-76)	Tomato (Brazil)	(7)	0	8	4	32
	B-203	Tomato (Fla.)	(1)	0	4	0	32
X. dieffenbachiae	B-401	Philodendron (Fla.)	(1)	0	0	0	0
X. corylina	B-408	Filbert (Ore.)	(5)	0	0	0	0
X. phaseoli	B-413 (XP-27)	Bean (Calif.)	(8)	0	0	0	0
X. malvacearum	B-412 (XM-5)	Cotton (Calif.)	(8)	0	0	0	0
X. maculifoliigardeniae	B-418	Gardenia (Fla.)	(9)	0	0	0	0
X. translucens	B-426 (537)	Wheat (S. D.)	(10)	0	0	0	0
X. translucens	B-430	Rye (Ga.)	(1)	0	0	0	0
X. incanae	B-425 (XI-4)	Unknown (Calif.)	(11)	0	0	0	0
Xanthomonas-'like'	B-404 (B-6440)	Human (Ga.)	(12)	0	0	0	0
X. pruni	B-102	Peach (Calif.)	(8)	0	0	0	0
X. poinsettiacola	B-422 (06F-F69)	Poinsettia (Fla.)	(9)	0	0	0	0
Unidentified bacteria	G-128	Cabbage, seed	(1)	0	0	0	0
	G-129	Cabbage, seed	(1)	0	0	0	0
	G-130	Broccoli, seed	(1)	0	0	0	0
	G-131	Cabbage, seed	(1)	0	0	0	0
	G-132	Cabbage, seed	(1)	0	0	0	0
Pseudomonas syringae	C-7 (B-3)	Peach (Calif.)	(13)	0	0	0	0
Escherichia coli	D-2 (U5-4)	Pig (Ga.)	(14)	0	0	0	0

[a]Titers are given as the reciprocal of the highest dilution of antiserum or γ globulin that caused fluorescence.

[b]The antirabbit globulin for indirect fluorescence was used at a 1:16 dilution only.

[c]Sources: (*1*) Author, Experiment, GA; (*2*) R. G. Grogan, University of California, Davis, CA; (*3*) D. Knosel, Universität Hamburg, Germany; (*4*) D. Dye, Dept. Scientific Industrial Research, Auckland, New Zealand; (*5*) L. Moore, Oregon State University, Corvallis, OR; (*6*) M. Sasser, University of Delaware, Newalk, DE; (*7*) C. F. Robbs, University of Rio de Janeiro, Rio de Janeiro, Brazil; (*8*) W. Schnathorst, University of California, Davis, CA; (*9*) J. Miller, State Department of Agriculture, Gainsville, FL; (*10*) J. Otta, South Dakota State University, Brookings, SD; (*11*) D. Hildebrand, University of California, Berkeley, CA; (*12*) P. Riley, U.S. Center for Disease Control, Atlanta, GA; (*13*) H. English, University of California, Davis, CA; (*14*) W. Ewing, U.S. Center for Disease Control, Atlanta, GA.

RESULTS

The molar F/P ratios (6) of the conjugates to ribosomes of *X. campestris* B-1 and *X. vesicatoria* B-215 used for the direct immunofluorescence tests were 8.0 and 9.1, respectively. Block titration tests showed that the optimum concentration of antirabbit globulin was 1:16 for both *X. campestris* and *X. vesicatoria*. In all cases the staining titers of the indirect IF tests were equal to or higher than the titers of the direct IF tests (Table 1). Both tests proved to be highly specific. Of the 24 other bacteria tested, including five starch-positive, mucoid, yellow-pigmented, nonpathogenic strains isolated from cabbage seeds on SX agar (11), only *X. vesicatoria* cross-reacted with *X. campestris*.

DISCUSSION

In the use of an immunological procedure as a diagnostic test, one important fact must be considered: the specificity of a diagnostic antiserum often depends on the specificity of the antigen injected. Ribosomes were chosen as antigens because they previously have been shown to be highly specific (9). Although nonspecific fluorescence tends to be more of a problem with the indirect than with the direct IF test (5), none was observed using antisera to 70S ribosomes. The cross-reactions between certain strains of *X. campestris* and *X. vesicatoria* agree with a previous report that the two closely related species share common antigens (9). Although some strains of *X. vesicatoria* could not be differentiated from *X. campestris*, the chances of isolating such strains from cabbage seeds would be extremely slight because *X. vesicatoria* is host-specific and is easily differentiated from *X. campestris* by its failure to hydrolyze starch on SX agar (11).

These studies have established the specificity of IF staining for identification of *X. campestris* isolated from crucifer seeds. Identification is accomplished more rapidly by this method than by pathogenicity tests. The investigation has demonstrated that the indirect IF is a reliable method of confirming the identification of *X. campestris* isolated from crucifer seeds and tentatively identified by colony morphology on agar media (10). These studies also show that *X. vesicatoria* can be identified quickly by indirect IF.

The indirect IF test is recommended over the direct IF test because of the higher staining titers achieved and because it is not necessary to evaluate the conjugate for dye content (F/P). Molar F/P ratios above 10 often result in nonspecific staining (5).

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