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Characterization of Monoclonal Antibodies Specific for *Erwinia carotovora* subsp. *atroseptica* and Comparison of Serological Methods for Its Sensitive Detection on Potato Tubers

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Seven monoclonal antibodies (MAbs) to *Erwinia carotovora* subsp. *atroseptica* have been produced. One, called 4G4, reacted with high specificity for serogroup I of *E. carotovora* subsp. *atroseptica*, the most common serogroup on potato tubers in different serological assays. Eighty-six strains belonging to different *E. carotovora* subsp. *atroseptica* serogroups were assayed. Some strains of serogroup XXII also reacted positively. No cross-reactions were observed against other species of plant pathogenic bacteria or 162 saprophytic bacteria from potato tubers. Only one strain of *E. chrysanthemi* from potato cross-reacted. A comparison of several serological techniques to detect *E. carotovora* subsp. *atroseptica* on potato tubers was performed with MAb 4G4 or polyclonal antibodies. The organism was extracted directly from potato peels of artificially inoculated tubers by soaking or selective enrichment under anaerobiosis in a medium with polypectate. MAb 4G4 was able to detect specifically 240 *E. carotovora* subsp. *atroseptica* cells per ml by indirect immunofluorescence and immunofluorescence colony staining and after soaking by ELISA-DAS (double-antibody sandwich enzyme-linked immunosorbent assay) after enrichment. The same amount of cells was detected by using immunolectrotransfer with polyclonal antibodies, and *E. carotovora* subsp. *atroseptica* and subsp. *carotovora* were distinguished by the latter technique. ELISA-DAS using MAb 4G4 with an enrichment step also efficiently detected *E. carotovora* subsp. *atroseptica* in naturally infected tubers and plants.

Three soft rot erwinias, *Erwinia carotovora* subsp. *atroseptica*, *E. carotovora* subsp. *carotovora*, and *E. chrysanthemi*, are commonly associated with potatoes. Although all three can cause tuber soft rot, only the *E. carotovora* subsp. *atroseptica* and *E. chrysanthemi* are the causal agents of blackleg in cool and warm regions, respectively (24). The detection of these bacteria on seed tubers is essential for the production of healthy seed crops because blackleg control relies primarily on the production of *E. carotovora* subsp. *atroseptica*-free and/or *E. chrysanthemi*-free seed.

Different methods are available for detection of erwinias. Growth on crystal violet pectate (CVP) at different temperatures allows the detection and differentiation of *E. carotovora* subsp. *atroseptica*, *E. carotovora* subsp. *carotovora*, and *E. chrysanthemi* (25). However, this procedure is not suitable for routine analysis of potato tubers, and it has been observed that not all *Erwinia* strains can be distinguished by this method (16).

Biochemical tests (5), protein electrophoresis (21), fatty acids (11), and isoenzyme profiles (13) have been applied to differentiate the three erwinias, but their use is restricted by the need to use pure cultures. PCR and restriction fragments length polymorphism are not being applied routinely for *E. carotovora* subsp. *atroseptica* detection (6).

Serological techniques are commonly used in some countries for *E. carotovora* subsp. *atroseptica* detection (32). They are appropriate in routine analysis provided that specific antibodies are available and the sensitivity of the assays is adequate, but neither condition is satisfied at present. Polyclonal antibodies (PAbs) against *E. carotovora* subsp. *atroseptica* usually cross-react with *E. carotovora* subsp. *carotovora* and epiphytic microflora of potato tubers. Monoclonal antibodies (MAbs) against *E. carotovora* subsp. *atroseptica* have been produced, but their value is limited by their lower affinity in enzyme-linked immunosorbent assay (ELISA) (10), or they have not been tested for detection on plant material (35).

The increasing interest in the use of serological procedures for the detection of *E. carotovora* subsp. *atroseptica* on potato tubers would benefit from a readily available supply of highly specific immunoglobulins with good affinity characteristics and sensitive detection methods. This report describes the production and characterization of MAbs against *E. carotovora* subsp. *atroseptica* and describes their specificity and suitability in different serological techniques for *E. carotovora* subsp. *atroseptica* detection on potato tubers, including a modified and sensitive ELISA method based on selective enrichment. Furthermore, the immunoelectrotransfer (IET) using PAbs that was used for specific *E. carotovora* subsp. *carotovora* and *E. carotovora* subsp. *atroseptica* differentiation in pure cultures (2) was also applied to potato tubers.

MATERIALS AND METHODS

Bacterial strains. Two hundred eleven strains from various culture collections were used. Eighty-two *E. carotovora* subsp. *atroseptica* and 69 *E. carotovora* subsp. *carotovora* strains isolated from potatoes in 10 different countries were included, and the serogroup of each strain was determined (12). In addition, the 40 strains representative of the serogroups described by De Boer were tested (Table 1). Three strains of *E. carotovora* subsp. *betavasculorum*, 5 of *E. chrysanthemi*, 1 of *E. rubrifaciens*, and 11 of different species of plant pathogenic bacteria were also used (Table 2).

Furthermore, 162 isolates of saprophytic microorganisms isolated in Spain from potato tubers and plants of different

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TABLE 1. Strains of *E. carotovora* subsp. *carotovora* and *E. carotovora* subsp. *atroseptica*

Subspecies	Strain or serogroup homolog	Serogroup[a]	Collection or source[b]	Origin
	Strains			
atroseptica	537.2, 573.3, 718.7, 718.1, 546.5	I	IVIA	Spain
	546.2, 722.5, 799.7, 578.3, 581.1	I	IVIA	Spain
	607.6	I	IVIA	Spain
	836.7	XXII	IVIA	Spain
carotovora	522.3, 837.3, 849.2	III	IVIA	Spain
	766.9, 766.2	V	IVIA	Spain
	847.1	V	IVIA	Spain
	757.1	IX	IVIA	Spain
	571.6	XV	IVIA	Spain
	663	XVIII	IVIA	Spain
	734.6, 776.2	XXVIII	IVIA	Spain
	543.1, 584.9, 822.7	XXIX	IVIA	Spain
	556.1	XXX	IVIA	Spain
	578.1	XXXII	IVIA	Spain
	663.8	XXXV	IVIA	Spain
	718.6, 720.2	XXXIX	IVIA	Spain
	531.1	XL	IVIA	Spain
	558.6, 563.1, 719.1	—	IVIA	Spain
	734.1, 764.6, 786.5	—	IVIA	Spain
atroseptica	1002, 93, 98, 1044, 69, 8, 79	I	SCRI (M. Pérombelon)	Scotland
	1055, 41, 37, 52, 1015, 1001	I	SCRI (M. Pérombelon)	Scotland
	1058	XX	SCRI (M. Pérombelon)	Scotland
	1054	XXII, XXXV	SCRI (M. Pérombelon)	Scotland
carotovora	194, 195	III	SCRI (M. Pérombelon)	Scotland
	121	XI	SCRI (M. Pérombelon)	Scotland
	212	XXVIII	SCRI (M. Pérombelon)	Scotland
	225	XXII, XXVIII, XL	SCRI (M. Pérombelon)	Scotland
	466	—	SCRI (M. Pérombelon)	Scotland
atroseptica	30184, 30186, 30185	I	DSM	Germany
	1419.86, 4196.75, 3467.86	I	ICMP	New Zealand
	4399.75, 4398.75	I	ICMP	New Zealand
	3468.73	—	ICMP	New Zealand
	KR 12, KR 1, KR 3, KR 31, KR 16	I	IZB (M. Arsenijéric)	Yugoslavia
	KR 21	XX	IZB (M. Arsenijéric)	Yugoslavia
	KR 155, KR 169, KR 20, KR 30	—	IZB (M. Arsenijéric)	Yugoslavia
	723, 161, 290	I	IPO (J. van Vuurde)	The Netherlands
	144	—	IPO (J. van Vuurde)	The Netherlands
	435, 433, 2043, 549, 309, 1277	I	NCPPB	England
	1743	XXII	NCPPB	England
	432	XXII, XXXV	NCPPB	England
	NO74, 81/N1, 81/N11.1, 81/N61, 81/N66	I	RAC (O. Cazelles)	Switzerland
	84/178, 85/5501, 89/42	—	RAC (O. Cazelles)	Switzerland
	86.14.11, 86.20, 87.7, 87.13, 88.1	I	INRA (S. Priou)	France
	88.22, 88.24, 88.30, 88.33, 88.45	I	INRA (S. Priou)	France
	SF 1.1, SF 18.2	—	INRA (S. Priou)	France
	Serogroup homologs			
atroseptica	31	I	S. H. De Boer	United States
carotovora	21	II	S. H. De Boer	The Netherlands
	71	III	S. H. De Boer	The Netherlands
	190	IV	S. H. De Boer	United States
	26	V	S. H. De Boer	The Netherlands
	189	VI	S. H. De Boer	United States
	68	VII	S. H. De Boer	United States
	62	VIII	S. H. De Boer	The Netherlands
	63	IX	S. H. De Boer	The Netherlands
	61	X	S. H. De Boer	The Netherlands
	193	XI	S. H. De Boer	Canada
	67	XII	S. H. De Boer	United States
	59	XIII	S. H. De Boer	The Netherlands
	65	XIV	S. H. De Boer	The Netherlands
	23	XV	S. H. De Boer	The Netherlands

Continued on following page

TABLE 1—*Continued*

Subspecies	Strain or serogroup homolog	Serogroup[a]	Collection or source[b]	Origin
	94	XVI	S. H. De Boer	Canada
	92	XVII	S. H. De Boer	Canada
atroseptica	6	XVIII	S. H. De Boer	Canada
carotovora	194	XIX	S. H. De Boer	Canada
atroseptica	196	XX	S. H. De Boer	Canada
carotovora	197	XXI	S. H. De Boer	Canada
atroseptica	198	XXII	S. H. De Boer	Canada
carotovora	207	XXIII	S. H. De Boer	Canada
	208	XXIV	S. H. De Boer	Canada
	210	XXV	S. H. De Boer	United States
	211	XXVI	S. H. De Boer	United States
	360	XXVII	S. H. De Boer	United States
	365	XXVIII	S. H. De Boer	United States
	380	XXIX	S. H. De Boer	United States
	209	XXX	S. H. De Boer	Canada
	477	XXXI	S. H. De Boer	United States
	476	XXXII	S. H. De Boer	United States
	474	XXXIII	S. H. De Boer	United States
	475	XXXIV	S. H. De Boer	United States
	499	XXXV	S. H. De Boer	United States
	497	XXXVI	S. H. De Boer	United States
	498	XXXVII	S. H. De Boer	United States
	500	XXXVIII	S. H. De Boer	United States
	518	XXXIX	S. H. De Boer	United States
	517	XL	S. H. De Boer	United States

[a] —, unknown serogroup (no reaction with the 40 antisera assayed representative of the serogroups).

[b] IVIA, Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain; SCRI, Scottish Crop Research Institute, Dundee, Scotland; DMS, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany; ICMP, International Collection of Micro-organisms from Plants, Auckland, New Zealand; IZB, Institut za Zastitu Bilja, Novi Sad, Yugoslavia; IPO, Research Institute for Plant Protection, Wageningen, The Netherlands; NCPPB, National Collection of Plant Pathogenic Bacteria, Hertfordshire, England; RAC, Station Fédérale de Recherches Agronomiques de Changins, Nyon, Switzerland; INRA, Institut National de la Recherche Agronomique, Rennes, France.

origins were examined. They were selected on the basis of colony morphology, Gram strain reaction, oxidase production, fermentative reaction with glucose in Hugh and Leifson medium (14), and pectate utilization (28).

Bacterial cultures were grown on King's B medium (19). Suspensions of 10^9 CFU/ml in sterile water were prepared and frozen at −20°C. They were used to study the specificity of MAbs in indirect ELISA (ELISA-I) and as controls. Suspensions from 24-h cultures were used to inoculate potato tubers.

Production and characterization of MAbs. BALB/c mice were immunized by intraperitoneal injection of living whole cells of *E. carotovora* subsp. *atroseptica* 1001, serogroup I (kindly provided by M. Pérombelon, Scottish Crop Research Institute, Dundee, Scotland). Hybridization of spleen cells was conducted by standard procedures (20, 23). Screening for the presence of antibodies was performed by an ELISA-I biotin-streptavidin system against the homologous strain (see below). A reaction was considered positive when the optical density reading was three times that obtained with a hybridoma culture supernatant of *Xylophilus ampelinus*, used as a control. Specific antibody-secreting hybridomas were cloned under conditions of limited dilution, using feeder layers (27). Cloning was repeated three times, and established hybrids were grown in hypoxanthine-thymidine medium. MAb isotypes were determined in culture supernatant by a commercial kit based on immunodiffusion (The Binding Site, Ltd., Birmingham, England). Ascitic fluid was produced (34), and MAbs were purified by affinity chromatography with protein A (Beckman).

Biotin conjugation. A solution containing 1 mg of purified MAb 4G4 per ml of 0.1 M sodium borate buffer (pH 8.8) was prepared to biotinylate antibodies with *N*-hydroxysuccinamide biotin (Sigma). The biotin was added in an amount equal to one-fifth of the weight of the immunoglobulins used. The *N*-hydroxysuccinamide biotin was previously dissolved in a volume of dimethyl sulfoxide equal to the volume of the immunoglobulins divided by 10. The solution was incubated for 4 h at room temperature, 20 μl of 1 M NH_4Cl per mg of immunoglobulins was added, and the solution was incubated for 10 min at room temperature. The uncoupled biotin was removed by 24 h of dialysis against 0.1 M phosphate-buffered saline (PBS; pH 7.2 to 7.4).

Production of PAbs. Antiserum was prepared from a rabbit (Californian × New Zealander) by immunization with cells of *E. carotovora* subsp. *atroseptica* 1001 dialyzed against glutaraldehyde for 3 h followed by dialysis against PBS for 24 h at 4°C. Immunization and antiserum production were done as described by Alarcón et al. (2), and immunoglobulins from the antiserum were purified.

Specificity of MAbs and PAbs. The specificities of the antibodies were evaluated in the ELISA-I biotin-streptavidin system (see below). Polystyrene microplates (Polisorp; Nunc) were coated with suspensions of 10^8 CFU of different bacterial species per ml in sodium carbonate buffer (0.05 M, pH 9.6) (Tables 1 and 2). Specificity was also tested against 162 isolates of potato saprophytic flora. MAb 4F6 (kindly provided by S. De Boer, Agriculture Canada Research Station, Vancouver, British Columbia, Canada) was used when required, for comparison.

Inoculation of plant material and extraction of *E. carotovora* subsp. *atroseptica*. The sensitivities of different serological techniques for *E. carotovora* subsp. *atroseptica* detection in plant material were evaluated by using potato tubers of variety Desirée. The tubers were checked on CVP for viable *E. carotovora* subsp. *atroseptica* before inoculation. They were

TABLE 2. Phytopathogenic bacteria

Strain	Species	Collection[a]
2795	*Erwinia carotovora* subsp. *betavasculorum*	NCPPB
2792	*Erwinia carotovora* subsp. *betavasculorum*	NCPPB
2794	*Erwinia carotovora* subsp. *betavasculorum*	NCPPB
2288	*E. chrysanthemi*	NCPPB
2467	*E. chrysanthemi*	NCPPB
2015	*E. chrysanthemi*	NCPPB
4367	*E. chrysanthemi*	NCPPB
597	*E. chrysanthemi*	NCPPB
818.8a	*Clavibacter michiganensis* subsp. *michiganensis*	IVIA
030.Sa	*Clavibacter michiganensis* subsp. *michiganensis*	IVIA
749.21	*Clavibacter michiganensis* subsp. *michiganensis*	IVIA
Na 2.2A	*Clavibacter michiganensis* subsp. *michiganensis*	IVIA
2437	*Agrobacterium tumefaciens*	NCPPB
33970	*Agrobacterium tumefaciens*	ATCC
2445	*Pseudomonas corrugata*	NCPPB
580c.77	*Pseudomonas corrugata*	IVIA
884.4a	*Xanthomonas campestris* pv. *juglandis*	IVIA
351.20	*Xanthomonas fragariae*	IVIA
G4	*Xylophilus ampelinus*	IVIA
29291	*E. rubrifaciens*	ATCC

[a] NCPPB, National Collection of Plant Pathogenic Bacteria, Hertfordshire, England; IVIA, Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain; ATCC, American Type Culture Collection, Rockville, Md.

washed with tap water and disinfected in sodium hypochlorite (2.5% active sodium hypochlorite) solution for 1 h, rinsed with ozonized water, dried, and stored at 4°C until use. The tubers were inoculated by vacuum infiltration at −86,000 lb/in^2 for 20 min by immersion in aqueous suspensions containing 10^1 to 10^{10} CFU of *E. carotovora* subsp. *atroseptica* 1001 and 10^8 to 10^{10} CFU of *E. carotovora* subsp. *carotovora* 194 per ml. The tubers were then dried and stored at 4°C for 15 days.

Three erwinia extraction procedures were compared. In each method, 2 g of potato peel obtained by peeling with a knife and 4 ml of buffer were used. In extraction method 1, the peels were cut in small 5-mm segments before soaking in buffer at room temperature for 1 h with gentle shaking. In method 2, the peels were blended in a Polytron homogenizer (Kinematica). In method 3, the peels were treated as in method 1 before enrichment in the same volume of double-PEM (DPEM) medium and incubation under anaerobic conditions for 48 h at 25°C. To prepare DPEM medium, $MgSO_4$ (0.64 g), $(NH_4)_2SO_4$ (2.16 g), and K_2PO_4H (2.16 g) were dissolved separately in 300 ml of distilled water. Then the $MgSO_4$ and $(NH_4)_2SO_4$ solutions were mixed and added to the K_2PO_4H solution, distilled water was added to 1 liter, 3.4 g of sodium polypectate (Bullmer Ltd., Plough Lane, Hereford, England) dissolved in 5 ml of ethanol was added, and the pH was adjusted to 7.2 before autoclaving. Three buffers also were compared with the three extraction methods: (i) PBS ($Na_2PO_4 \cdot 12H_2O$ [2.7 g], $NaPO_4H_2 \cdot 2H_2O$ [0.4 g], NaCl [8 g], distilled water [1 liter] [pH 7.2]) plus 2% polyvinylpyrrolidone (molecular weight, 10,000); (ii) PBS plus 0.2% sodium diethyldithiocarbamate (DIECA); and (iii) distilled water plus 1.5% dithiothreitol.

To compare the efficiencies of the disinfection and extraction methods, populations of *E. carotovora* subsp. *carotovora* and *E. carotovora* subsp. *atroseptica* in the different samples were determined by dilution plating on CVP. Plates were incubated at 25 and 33.5°C to distinguish *E. carotovora* subsp. *atroseptica* 1001 and *E. carotovora* subsp. *carotovora* 194 (25). Both strains were previously checked for cavity formation on CVP at these temperatures.

Slide agglutination. Two hundred microliters of ascitic fluid of MAb 4G4 was mixed with a pure culture of *E. carotovora* subsp. *atroseptica* 1001 on a slide. *X. ampelinus* 60.7 was used as a negative control. The agglutination was observed under a binocular microscope after 1 min.

Comparison of serological techniques to detect *E. carotovora* subsp. *atroseptica*. Immunofluorescence (IF), immunofluorescence colony staining (IFC), ELISA-I, double-antibody sandwich ELISA (ELISA-DAS), and IET were compared for the detection of *E. carotovora* subsp. *atroseptica* in pure culture suspensions or in potato peel extracts. Peels from 10 inoculated potato tubers were collected and pooled. Two-gram lots were subjected to the three extraction methods and buffers described above. Noninoculated tubers were used as a control. Cell suspensions of *E. carotovora* subsp. *atroseptica* 1001 at dilutions from 10^1 to 10^8 CFU/ml were used as a positive control in all techniques. A suspension of *X. ampelinus* 60.7 at 10^8 CFU/ml was used as a negative control.

Indirect IF and IFC. Indirect IF was done as described previously (8) but with 80 μg of biotinylated MAb 4G4 per ml of PBS and streptavidin conjugated with fluorescein isothiocyanate (Boehringer Mannheim). IFC was done by the standard procedure (31, 33) but with the same conjugate and MAb concentrations as in IF. Four replications of each sample were made.

ELISA-I. Polystyrene plates (Polysorp F96; Nunc) were coated with 200 μl of the test sample per well after a soaking or blending extraction procedure. Four wells in two plates were used for each sample. When the enrichment method was used, 100 μl of each sample after soaking or blending was mixed in wells of the microplate with 100 μl of DPEM and incubated for 48 h at 25°C in anaerobiosis. The plates without enrichment were incubated 16 h at 4°C. For the ELISA-I technique, MAb 4G4 was added at 0.25 μg/ml in PBS. The reaction was revealed by goat anti-mouse immunoglobulins conjugated with biotin and streptavidin-alkaline phosphatase (Boehringer Mannheim). The readings of the hydrolyzed substrate for alkaline phosphatase were made at 405 nm at 30, 60, and 90 min in a Titertek Multiskan (Flow) reader. Optical density values greater than twice those of the negative control (*X. ampelinus*, 10^8 CFU/ml) were considered positive.

ELISA-DAS. Polystyrene microplates (Maxisorp F96; Nunc) were used. The wells were coated with 200 μl of immunoglobulins of GA-1001 antiserum (2) or of MAb 4G4 at 1 μg/ml. The plates were incubated for 4 h at 37°C and washed. Then 200 μl of the different peel extracts (after soaking or blending) was added in each of four wells of two microplates; the plates were incubated for 16 h at 4°C or enriched in DPEM as described above and then washed. The biotin conjugate of 4G4 was added at 0.1 μg of immunoglobulins per ml in PBS, incubated for 2 h at 37°C, and washed. The final step and readings were as for ELISA-I.

Electrophoresis and IET. Western blotting (immunoblotting) followed by ELISA-I (i.e., IET) was done with antiserum GA-1001. One milliliter of peel extracts prepared as in the soaking method was centrifuged at 17,000 × *g* for 20 min at 4°C. The pellet was resuspended in 0.5 ml of a preparation containing 7 volumes of distilled water and 3 volumes of denaturing mixture (2) and boiled for 10 min. Sixty-microliter volumes of the samples were loaded in gels, and electrophoresis was carried out as described previously (2). When the enrichment method was used, 1 ml of the enriched sample was centrifuged and processed as described above.

Analysis of commercial plant material and tubers. A total of 144 potato samples (60 samples of potato tubers and 84 potato stems of different varieties) were simultaneously analyzed by

direct isolation on plates of CVP medium incubated at 27 and 33.5°C, using conventional ELISA-DAS and ELISA-DAS with previous enrichment on DPEM as described above. To analyze material, sap expressed from potato stems was diluted 1:2 in PBS, and peel extract from tubers was prepared by soaking in PBS plus 0.2% DIECA as described for the artificially inoculated material.

RESULTS

Production of antibodies, specificity, and biotin conjugation. Using hybridoma technology, we produced seven MAbs, of which three (4G4, 1E4, and 3D11) showed about the same pattern of reaction with different bacteria. One, 4G4, was selected for its ability to react and its specificity for *E. carotovora* subsp. *atroseptica*. Two others (5F7 and 2H8) exhibited a wide range of reaction with different *Erwinia* serogroups and some saprophytic bacteria. MAb 4G4 was able to recognize epitopes on *E. carotovora* subsp. *atroseptica* 1001 (serogroup I) and did not react with *E. carotovora* subsp. *carotovora* 194. It was isotype IgG3, K. The reacting epitope was probably lipopolysaccharide (LPS) because a reaction with an extract of *E. carotovora* subsp. *atroseptica* 1001 LPS was observed by IET (data not shown). The ascitic fluid (diluted 1/1,000) was able to induce clear agglutinates when mixed with *E. carotovora* subsp. *atroseptica* 1001 but not when mixed with *X. ampelinus*. After purification from ascitic fluid, MAb 4G4 was used at 0.2 μg of immunoglobulins per ml in ELISA-I.

Table 3 shows the reaction of MAb 4G4, which recognized 66 strains of *E. carotovora* subsp. *atroseptica* and 1 strain of *E. chrysanthemi*. It reacted with *E. carotovora* subsp. *atroseptica* strains of serogroups I and XXII but not with *E. carotovora* subsp. *atroseptica* strains of serogroups XVIII and XX or with the untyped strains. *E. carotovora* subsp. *atroseptica* strains considered atypical according to biochemical tests showed different reactions with MAb: most of the serogroup I strains reacted with 4G4, but those of unknown serogroup did not. A reaction was observed with one *E. chrysanthemi* strain. MAb 4G4 did not react with 69 *E. carotovora* subsp. *carotovora* potato strains, 3 *E. carotovora* subsp. *betavasculorum* strains, 4 *E. chrysanthemi* strains, 12 strains of various plant pathogenic species, and 162 isolates of saprophytic flora of potato.

The dilution endpoint of the biotinylated 4G4 antibody was 1/256,000 by ELISA-DAS. It was used at 0.1 μg/ml in PBS.

Inoculation of plant material. Results obtained by viable count on noninoculated tubers show that 100 to 200 pectolytic colonies identified as *E. carotovora* subsp. *carotovora* by specific biochemical and physiological tests (5) appeared on CVP plates. A greater number of *E. carotovora* subsp. *atroseptica* cells was obtained with enrichment (about 10^8 to 10^{10} CFU/ml of peel extract) than with soaking or blending extraction procedures (about 10^5 to 10^6 CFU/ml). No statistically significant differences were detected between soaking or blending, but enrichment was significantly different. The best buffers were PBS plus 0.2% DIECA and PBS plus 2% polyvinylpyrrolidone. Dithiothreitol was significantly less efficient. The three methods of extraction (soaking, blending, and enrichment) were compared by different serological techniques using PBS buffer plus 0.2% DIECA.

Comparison of serological techniques. The sensitivity of each of the techniques assayed is shown in Table 4. Only small differences in sensitivity were observed between the results obtained after extraction by soaking or blending potato peels. Only results after soaking extraction are shown in Table 4.

In IF, fluorescein-stained cells of *E. carotovora* subsp. *atroseptica* 1001 were detected in all the samples containing 240 or more cells of *E. carotovora* subsp. *atroseptica* 1001. None were found in samples containing *E. carotovora* subsp. *carotovora* or in noninoculated samples. Similar results were obtained in IFC. Fluorescein-stained colonies were observed in all samples inoculated with *E. carotovora* subsp. *atroseptica* but not in the noninoculated samples.

ELISA results are shown in Fig. 1 and Table 4. Results in ELISA-DAS, using biotinylated MAb 4G4 as the conjugate, were not statistically different with PAbs or MAb 4G4 used for coating the plates. Only results obtained with the MAb for coating are shown. The sensitivity of the detection in ELISA-DAS was close to 10^5 cells per ml in pure culture. After soaking of the samples, the method detected *E. carotovora* subsp. *atroseptica* 1001 in extracts containing 1.6×10^5 or more CFU/ml. The highest sensitivity in ELISA-DAS was obtained after enrichment of the peel samples. The enrichment was not observed in the controls of *E. carotovora* subsp. *atroseptica* because frozen cells were used. Nevertheless, enrichment of fresh pure cultures of *E. carotovora* subsp. *atroseptica* with PBS as a buffer was able to detect 10^2 or more CFU/ml (data not shown). The method was able to detect 2.4×10^2 or more CFU of *E. carotovora* subsp. *atroseptica* per ml in this experiment. Noninoculated samples and those inoculated with *E. carotovora* subsp. *carotovora* 194 were negative. ELISA-I after enrichment was able to detect *E. carotovora* subsp. *atroseptica* only in samples with 1.6×10^5 CFU/ml and higher concentrations.

The results obtained by IET are also shown in Table 4. After soaking of the samples, *E. carotovora* subsp. *atroseptica* was detected when the samples had 3.8×10^4 or more CFU/ml. The highest sensitivity was also observed in enriched samples because *E. carotovora* subsp. *atroseptica* was detected at all the concentrations studied and the mobility of the specific *E. carotovora* subsp. *atroseptica* and *E. carotovora* subsp. *carotovora* bands allowed differentiation of the two subspecies (2). It was also possible to detect *E. carotovora* subsp. *carotovora* in the noninoculated samples and to distinguish *E. carotovora* subsp. *carotovora* and *E. carotovora* subsp. *atroseptica* at the same time in some samples. These *E. carotovora* subsp. *carotovora* cells were part of the natural microflora of the potato tubers. In the sample with 100 CFU of *E. carotovora* subsp. *atroseptica* per ml, the characteristic band of *E. carotovora* subsp. *atroseptica* was observed but with low intensity. Comparison of the sensitivities of the different techniques studied, using the same extracts of potato peels, shows that IF, IFC, and ELISA-DAS or IET using enrichment were the most sensitive (Table 4).

Analysis of commercial plants and tubers. Table 5 show the results obtained in the analysis of 144 samples of plants and tubers from commercial fields of different origins. Some of the samples were free of *E. carotovora* subsp. *atroseptica*, but others were found to be infected by the bacterium. The results show the higher detection ability of ELISA-DAS with previous enrichment, in comparison with direct plating on CVP and conventional ELISA-DAS.

False negatives were observed after direct plating on CVP because *E. carotovora* subsp. *atroseptica* was detected by plating after enrichment. False negatives were also observed by conventional ELISA-DAS because *E. carotovora* subsp. *atroseptica* was detected by direct plating or after ELISA-DAS enrichment.

DISCUSSION

This report describes the production and characterization of MAbs raised against *E. carotovora* subsp. *atroseptica* and the

TABLE 3. Reactions of MAb 4G4 with strains of *E. carotovora* subspp. *atroseptica* and *carotovora* of several serogroups in ELISA-I

Strain	Serogroup(s)[a]	4G4 reactivity
E. carotovora subsp. *atroseptica*		
537.2	I	+
573.3	I	+
546.5	I	+
546.2	I	+
578.3	I	+
581.1	I	+
718.7	I	+
718.1	I	+
722.5	I	+
799.7	I	+
607.6	I	+
836.7	XXII	+
1002	I	+
93	I	+
1054[b]	XXII, XXXV	−
69	I	+
1055	I	+
41	I	+
1058[b]	XX	−
1015	I	+
98	I	+
1044[b]	I	−
8	I	+
79	I	+
37	I	+
52	I	+
30184	I	+
30185	I	+
30186	I	+
3468.73[b]	—	+
1419.86	I	+
4399.75	I	+
4398.75	I	+
4196.75	I	+
3467.86	I	+
KR 12	I	+
KR 21[b]	XX	−
KR 31	I	+
KR 16	I	+
KR 155[b]	—	−
KR 169[b]	—	−
KR 1	I	+
KR 20[b]	—	−
KR 30[b]	—	−
KR 3	I	+
723	I	+
161	I	+
144	—	+
290	I	+
435	I	+
432[b]	I	−
549	I	+
309	I	+
433	I	+
31	I	+
6[b]	XVIII	−
196[b]	XX	−
198	XXII	+
2043	I	+
1277	I	+
1743	XXII	+
NO74	I	+
81/N1	I	+
81/N111	I	+
81/N61	I	+
81/N66	I	+
84/178[b]	—	−
E. carotovora subsp. *carotovora*		
522.3	III	−
531.1	XL	−
543.1	XXIX	−
556.1	XXX	
558.6	—	−
563.1	—	−
571.6	XV	−
578.1	XXXII	−
584.9	XXIX	−
663.8	XXXV	−
663	XVIII	−
718.6	XXXIX	−
719.1	—	−
720.2	XXXIX	−
734.1	—	−
734.6	XXVIII	−
764.6	—	−
766.9	V	−
766.2	V	−
776.2	XXVIII	−
757.1	IX	−
786.5	—	−
822.7	XXIX	−
837.3	III	−
847.1	V	−
849.2	III	−
225	XXII, XXVII, XL	−
121	XI	−
212	XXVIII	−
195	III	−
466	—	−
21	II	−
71	III	−
190	IV	−
26	V	−
189	VI	−
68	VII	−
62	VIII	−
63	IX	−
61	X	−
193	XI	−
67	XII	−
59	XIII	−
65	XIV	−
23	XV	−
94	XVI	−
92	XVII	−
194	XIX	−
197	XXI	−
207	XXIII	−
208	XXIV	−
210	XXV	−
211	XXVI	−
360	XXVII	−
365	XXVIII	−
380	XXIX	−
209	XXX	−
477	XXXI	−
476	XXXII	−
474	XXXIII	−
475	XXXIV	−
499	XXXV	−
497	XXXVI	−
498	XXXVII	−
500	XXXVIII	
518	XXXIX	−

Continued on following page

TABLE 3—*Continued*

Strain	Serogroup(s)[a]	4G4 reactivity	Strain	Serogroup(s)	4G4 reactivity
85/551[b]	—	−	517	XL	−
			E. carotovora subsp. *betavasculorum*		
89/42[b]	—	−	2795		−
86.14.11	I	+	2792		−
86.20	I	+	2794		−
			E. chrysanthemi		
87.7	I	+	2288		−
87.13	I	+	2467		−
88.1	I	+	2015		−
88.22	I	+	4367		+
88.24	I	+	597		−
88.30	I	+			
88.33	I	+			
88.45	I	+			
SF 1.1[b]	—	−			
SF 18.2[b]	—	−			

[a] —, unknown serogroup (no reaction with the 40 antisera of the 40 serogroups assayed).
[b] Atypical strain according to biochemical tests.

ability of a selected MAb (4G4) to react in different serological assays directed to improve the sensitivity of detection.

Other available MAbs for *E. carotovora* subsp. *atroseptica* detection have been described (10, 35). One was specific for serogroups I and XXII of *E. carotovora* subsp. *atroseptica* and could be used in ELISA only as an enzyme conjugate, not for coating plates. Furthermore, its reaction with the homologous bacteria in ELISA was appreciably weaker than with PAbs, and the authors concluded that MAbs to other antigens would be more useful in ELISAs for detecting tuber contamination (10). Recently, a MAb claimed to be specific to *E. carotovora* subsp. *atroseptica* serogroup I has been produced (35), but it has been assayed against only three *E. carotovora* subsp. *atroseptica* strains.

In our work, whole cells of *E. carotovora* subsp. *atroseptica* serogroup I were used to immunize mice and for initial screening, but the MAbs obtained were also able to react with other serogroups. For example, MAbs 4G4 and 3D11 were specific (with the same pattern of reaction and ability to react) to serogroup I and some strains of serogroup XXII. MAbs 5F7 and 2H8 reacted not only with *E. carotovora* subsp. *atroseptica* but also with most other erwinias as well as with some saprophytic bacteria. These results show the presence of common epitopes in erwinias and other bacterial species, as already shown with PAbs. Nevertheless, the observation that the selected MAb, 4G4, reacted only with *E. carotovora* subsp. *atroseptica* serogroup I and with some strains of serogroup XXII suggests the presence of a common, well-conserved, and frequent antigenic determinant in serogroup I. Results obtained with MAb 4F6 from S. De Boer were very similar (data not shown). It is likely that this is an LPS epitope and confirms the high immunological potential of LPS of these bacteria and the similarity in the O side chains of the two serogroups reported by other authors (10). Serological cross-reactivity observed between *E. carotovora* subsp. *atroseptica* strains of serogroup I and *E. carotovora* subsp. *carotovora* strains of serogroup II previously reported (9) was not found when MAb 4G4 was used.

TABLE 4. Detection of *E. carotovora* subsp. *atroseptica* in different artificially inoculated samples by indirect IF, IFC, ELISA-DAS, ELISA-I, and IET[a]

Sample[b]	CFU/ml[c]	IF, soaking[d]	IFC, soaking	ELISA-DAS		ELISA-I		IET	
				Soaking	Enrichment[e]	Soaking	Enrichment	Soaking	Enrichment
0	1.0×10^3 (Ecc)	−	−	−	−	−	−	−	+Ecc
1	2.4×10^2 (Eca)	+	+	−	+	−	−	−	+Ecc, Eca
2	3.1×10^2 (Eca)	+	+	−	+	−	−	−	+Eca
3	7.0×10^3 (Eca)	+	+	−	+	−	−	−	+Eca
4	3.0×10^4 (Eca)	+	+	−	+	−	−	−	+Ecc, Eca
5	1.6×10^5 (Eca)	+	+	+	+	−	+	−	+Eca
6	3.0×10^5 (Eca)	+	+	+	+	+	+	+Eca	+Eca
7	6.2×10^7 (Eca)	+	+	+	+	+	+	+Eca	+Eca
8	3.0×10^8 (Eca)	+	+	+	+	+	+	+Eca	+Eca
9	1.8×10^4 (Ecc)	−	w+[f]	−	−	−	−	+Ecc	+Ecc
10	3.8×10^4 (Ecc)	−	w+	−	−	−	−	+Ecc	+Ecc
11	1.1×10^5 (Ecc)	−	w+	−	−	−	−	+Ecc	+Ecc

[a] MAb 4G4 was used for IF, IFC, ELISA-I, and ELISA-DAS. PAbs were used for IET.
[b] Sample 0 was disinfected, uninoculated tubers. Samples 1 to 8 were disinfected tubers inoculated with *E. carotovora* subsp. *atroseptica* (Eca) 1001 at different concentrations. Samples 9 to 11 were disinfected tubers inoculated with *E. carotovora* subsp. *carotovora* (Ecc) 194.
[c] Determined by viable counts on CVP at the time of sampling for serological analysis.
[d] Soaking of the peel extract in PBS–0.2% DIECA for 1 h.
[e] Enrichment of the peel extract in DPEM medium.
[f] w+, weak fluorescence.

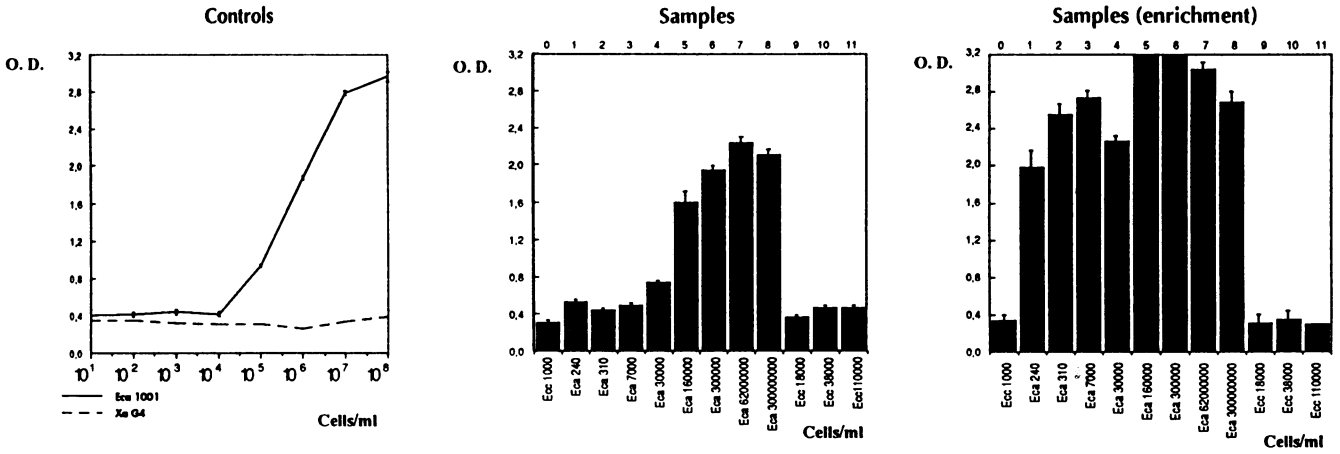

FIG. 1. Detection of *Erwinia carotovora* subsp. *atroseptica* (Eca) by ELISA-DAS and ELISA-DAS after enrichment of the peel extract, using MAb 4G4. Controls were dilutions of pure cultures of *E. carotovora* subsp. *atroseptica* 1001 and of *X. ampelinus* (Xa). Sample 0 was uninoculated, disinfected tubers; samples 1 to 8 were disinfected tubers inoculated with *E. carotovora* subsp. *atroseptica* 1001 at different concentrations; samples 9 to 11 were disinfected tubers inoculated with *E. carotovora* subsp. *carotovora* (Ecc) 194.

The selected MAb, 4G4, did not react with *E. carotovora* subsp. *carotovora*, *E. carotovora* subsp. *betavasculorum*, or other species of plant pathogenic bacteria or with the epiphytic flora of the potato tubers and plants. The reaction observed against one *E. chrysanthemi* isolate could be caused by a common epitope between some strains of *E. chrysanthemi* and *E. carotovora* subsp. *atroseptica* serogroup I strains (29). *E. chrysanthemi* has not been reported in many potato-producing countries in Europe (Ireland, Scotland, Spain, Portugal, etc.).

Most *E. carotovora* subsp. *atroseptica* isolated from diseased plants in cooler climates belong to serogroups I, XVIII, XX, and XXII; of these, more than 80% of *E. carotovora* subsp. *atroseptica* isolates are of serogroup I (22), with some exceptions (18). The other serogroups have been found at low frequency (7). It is notable that *E. carotovora* subsp. *atroseptica* strains in serogroup I but not in serogroup XVIII, XX, or XXII were found to be biochemically and serologically homogeneous (12). This could explain why MAb 4G4 reacted with only three strains of serogroup XXII. Serogroups XVIII and XX have homogeneous LPS, but their patterns differ from those of serogroups I and XXII (12).

The ability of MAb 4G4 to react with high affinity in all of the serological methods examined showed that it could have a number of different uses. It was serologically reactive in agglutination, indirect IF, IFC, ELISA-I, and ELISA-DAS as both a primary and a secondary antibody simultaneously. The sensitive detection of *E. carotovora* subsp. *atroseptica* on tubers is also related to the efficiency of the extraction method used.

TABLE 5. Analysis of 144 potato samples healthy or naturally infected by *E. carotovora* subsp. *atroseptica*

Method	No. positive	No. negative	No. false negative	No. false positive
Direct plating on CVP at 25 and 33°C	80	64	13[a]	
ELISA-DAS	65[b]	79	28[a,b]	0
ELISA-DAS with previous enrichment	93[a]	51	0	0

[a] Confirmed by plating on CVP after enrichment.
[b] Confirmed by direct plating on CVP.

The use of a soaking procedure in a buffer with DIECA seems to be more efficient than in a method with dithiothreitol that was used by other authors (25). When the sensitivities of the different techniques to detect *E. carotovora* subsp. *atroseptica* in artificially inoculated tubers were examined, results obtained by IF and IFC showed that both techniques were sensitive, even without enrichment, and were able to detect about 10^2 CFU/ml of peel extract. However, in IFC, the intensity of the staining not only is related to the amount of the antigen on the cells as in IF but also depends on the position of the colonies in the dry agar film, which required constant focusing. The time required to read plates is generally less than in IF, but the assay requires 48 to 72 h to complete and the largest amount of antibodies.

ELISA has been used before by several authors for detection of *E. carotovora* subsp. *atroseptica* on potato tubers (4, 15, 26, 30, 36) but was generally considered not sensitive enough. Nonetheless, in some countries, such as The Netherlands, the technique is used with an antiserum against *E. carotovora* subsp. *atroseptica* serogroup I in routine analysis of potato seed tubers (32). The use of an enrichment step to increase the sensitivity of ELISA for *E. carotovora* subsp. *atroseptica* detection was previously proposed (3), and the medium of Kado and Hesket (17) was used in combination with polyclonal antibodies of undefined specificity to increase the sensitivity. In our experiments, enrichment was based on the preferential ability of the soft rot erwinias to metabolize polypectate under anaerobic conditions and was found to improve the sensitivity of all techniques. ELISA-DAS with enrichment is a very sensitive and specific technique, with a lower detection level of 10^2 to 10^3 cells per ml in the experiment with artificially inoculated tubers when MAb 4G4 was used. Antibody coating was apparently not affected by enrichment under anaerobic conditions, nor was growth of *E. carotovora* subsp. *atroseptica* by the presence of *E. carotovora* subsp. *carotovora* or the background bacteria. The ELISA-DAS with enrichment method has also been used successfully for *E. carotovora* subsp. *atroseptica* detection in naturally infected potato tubers and plants (1). The results show that this method is more efficient than CVP isolation for *E. carotovora* subsp. *atroseptica* detection.

The proposed method for routine analysis of potato tubers using ELISA-DAS for detection of *E. carotovora* subsp. *atroseptica* contamination should include two assays: one plate directly processed by conventional ELISA-DAS and another processed after enrichment. If both are positive, the sample will contain more than 10^5 CFU/ml of potato peel extract. If only the enrichment plate is positive, *E. carotovora* subsp. *atroseptica* numbers present will be between 10^2 and 10^5 CFU/ml. In this way, the ELISA-DAS biotin-streptavidin system using MAb 4G4 with enrichment on DPEM seems to be a useful technique for large-scale testing of potato tubers and plants because it is simple, specific, accurate, reproducible, and of high sensitivity.

The IET technique was described to distinguish *E. carotovora* subsp. *carotovora* and *E. carotovora* subsp. *atroseptica* by using pure cultures of the bacteria (2). In this study, it has been shown to be also useful in distinguishing the two subspecies in potato peel extracts. The technique is not adapted for routine use with a large number of samples but is sensitive in detecting 10^2 CFU/ml after enrichment and is specific for both erwinias. This technique has also recently been used (30) with potato peel extracts after immunomagnetic separation of bacteria from the samples. IET can be used to confirm doubtful ELISA results or to determine both subspecies if the number of samples is not very high.

Low specificity of antibodies and low sensitivity of some serological techniques are the most frequent problems observed in the practical use of serology for detection of plant pathogenic bacteria. Both may be avoided by using MAb 4G4 and the described techniques to detect *E. carotovora* subsp. *atroseptica* on potato tubers.

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