Serogroups of *Erwinia carotovora* Potato Strains Determined with Diffusible Somatic Antigens

S. H. De Boer, R. J. Copeman, and H. Vruggink

Research plant pathologist, Research Station, Agriculture Canada, 6660 N.W. Marine Drive, Vancouver, British Columbia, V6T 1X2; associate professor, Department of Plant Science, University of British Columbia, Vancouver, V6T 1W5; and research associate, Institute of Phytopathological Research, Wageningen, The Netherlands, respectively.

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ABSTRACT

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Antisera produced against selected potato strains of *Erwinia carotovora* were used to classify unknown strains of *E. carotovora* into serogroups. The basis of a serogroup was a reaction of identity with the homologous strain by double diffusion. The antigen responsible for precipitin band formation was heat stable and could be extracted with a conventional phenol-water extraction procedure for lipopolysaccharides. Approximately 83% of 1,001 strains from several culture collections could be classified in one of 18 serogroups that were established. In a survey of *E. carotovora* strains in British Columbia, five serogroups were found frequently; other serogroups were isolated rarely or not at all. Most strains identified as *E. carotovora* var. *atroseptica* belonged to one serogroup, but 14 strains belonged to a second serogroup which also accommodated two strains that were biochemically identified as *E. carotovora* var. *carotovora*. The two *E. carotovora* var. *atroseptica* serogroups were serologically related and one of these also was related to an *E. carotovora* var. *carotovora* serogroup.

Additional key words: bacterial soft rot, blackleg, serology, O antigen.

The results of recent studies on potato blackleg and soft rot caused by *Erwinia carotovora* var. *atroseptica* (van Hall) Dye (*Eca*) and *E. carotovora* var. *carotovora* (Jones) Dye (*Ecc*), respectively, have established that the potato seed piece is an important inoculum source (5,6,24). However, in several programs in which stem-cuttings were used to eliminate tuber-borne pathogens, a small percentage of plants became recontaminated with *Ecc* and occasionally with *Eca* (2,5,11,25,26). That evidence suggests that the seed piece is not the only source of inoculum. In Scotland, transmission of the blackleg organism from waste potato dumps by insects (11,15) and from infected fields via aerosols (12,13) has been reported. Soil also has been implicated as a possible inoculum source (2,21).

Ecological studies are needed to define more clearly the survival characteristics of *Eca* and *Ecc* during the intercrop season under different climatic and soil conditions. However, ecological and epidemiological studies have been hampered by the difficulty in identifying strains of these organisms. Although serological procedures are used routinely to identify strains of enteric bacteria (8,16), they have been used successfully with the soft rot coliform bacteria only to a limited extent (14,27,29,30).

Immunodiffusion and agglutination studies involving many strains have shown that *E. carotovora* is highly variable serologically (18), although broad agglutination groups could be identified among potato strains (7). Moreover, in several studies in which soft rot bacteria in potato tissue were detected by agglutination, immunodiffusion, or immunofluorescence, it became apparent that *Eca* was serologically homogeneous and that *Ecc* was more variable (1,9,32). A serotyping scheme for *E. carotovora* like that devised for other Enterobacteriaceae (8) would be useful for strain identification. The study of strain distribution in other hosts, natural habitats, and geographic locations would facilitate discovery of inoculum sources.

We report here the use of the Ouchterlony double diffusion method (22) for grouping potato strains of *E. carotovora* into serogroups on the basis of diffusible somatic antigens. A preliminary report has been presented (4).

MATERIALS AND METHODS

Bacterial cultures. Potato strains of *E. carotovora* were obtained from H.P. Maas-Geesteranus (Wageningen, The Netherlands), A. Kelman (Madison, WS), or collected by the authors. Potato strains as different as possible were selected by criteria such as origin and colony morphology. Cultures were tested for pectolytic activity and typical colony morphology on crystal violet pectate medium (CVP) (3) and ability to decay potato disks. Acid production from α-methyl-D-glucoside, production of reducing substances from sucrose, and ability to grow at 37 C were tested by the methods reported previously (5). To distinguish *E. carotovora* from other pectolytic *Erwinia* spp., phosphatase production was tested by growing cultures on Difco nutrient agar plus 0.5% sodium phenolphthalein diphosphate (Sigma Chemical Co., St. Louis, MO 63178) for 48 hr, then exposing the colonies to ammonium vapor for a few minutes; phosphatase-positive colonies turned pink (10). Cultures routinely were maintained in sterile distilled water at room temperature and grown on nutrient agar medium at 24 C.

Antisera production. Antisera were produced in rabbits against several different cell preparations and with differing injection schedules. For all preparations, bacterial cells were grown on nutrient agar slants at 24 C for 24 hr, suspended in distilled water, and washed two or three times by centrifugation and resuspension. The final concentration was adjusted to 10^9–10^{10} cells per ml.

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Antisera were produced against live, whole cells at Wageningen. For the initial injection, 2 ml of cell suspension were emulsified with an equal volume of Freund's incomplete adjuvant and injected subcutaneously in the neck region. After 3 wk a series of intravenous injections of 0.3, 0.5, 1.0 and 2.0 ml of cell suspension without adjuvant were given at 2- to 3-day intervals.

Antisera were produced against whole, glutaraldehyde-fixed, and heat-treated, fixed cells at the University of British Columbia. Thrice washed cells were fixed in glutaraldehyde by the method of Allan and Kelman (1) with or without prior heat treatment at 121 C for 2 hr. Rabbits were given six intramuscular injections of 0.5 ml cell suspension emulsified with an equal volume of Freund's incomplete adjuvant at weekly intervals or three intramuscular injections followed by two intravenous injections of 0.5 and 1.0 ml cell suspension without adjuvant.

Rabbits were bled from the marginal ear vein 1 wk after the final injection and at 1-wk intervals thereafter up to six bleedings. Additional intramuscular or intravenous injections were given when necessary to maintain titers. Titers were determined by a drop agglutination method in which a drop of each of 15 serial dilutions of antiserum was added to a drop of homologous bacterial suspension ($OD_{660\ nm} \simeq 0.15$) in plastic petri dishes. Agglutination was observed under a stereomicroscope at ×10 after 2 and 4 hr at room temperature.

Sera were stored frozen except for small samples for regular use which were stored at 3 C with merthiolate (1:10,000) or sodium azide (1:5,000) added as a preservative.

Immunodiffusion. Agar plates for double diffusion tests were prepared with 15 ml of 0.8% Oxoid or Difco purified agar, 0.85% NaCl and 200 ppm sodium azide; the medium was dispensed into plastic petri dishes (100 × 15 mm). Wells, 3 mm in diameter and 4 mm apart, were cut in sets of six peripheral wells surrounding a center well; 12 sets were cut in each plate. Bacterial cells for use in double diffusion were grown at 24 C for 24 hr on nutrient agar slants and harvested in 1 ml distilled water (cell concentration $\simeq 10^9$–10^{10} cells per milliliter). A drop of liquefied phenol was added to the cell suspension and thoroughly mixed. The center wells were filled with undiluted antisera; two wells on opposite sides of the center well were filled with the homologous cell suspension and the remaining four wells were filled with suspensions of unknown bacteria or other test preparations. The plates were incubated at room temperature for 24–48 hr before being examined for precipitin bands.

Antigen extraction. A conventional phenol-water extraction procedure for lipopolysaccharides (LPS) was used (33). Acetone-dried bacteria (0.1 g) were suspended in 10 ml distilled water, heated to 70 C, and an equal volume of liquefied phenol, preheated to 70 C, was added. The mixture was stirred for 15 min, cooled in an ice bath, and the phases were separated by centrifugation. The aqueous phase was retained and the pellet was extracted a second time. The extracts were pooled and dialized overnight against distilled water at 3 C.

TABLE 1. Agglutination titers (drop method) of antisera (first bleeding) produced in rabbits against untreated, glutaraldehyde-fixed, and fixed, heat-treated *Erwinia carotovora* cells

Strain number	Reciprocal of antiserum titer		
	Untreated cells	Fixed cells	Fixed, heat-treated cells
31	1,024	4,096	512
21	256		8
24	16,384	2,048	
190		4,096	32
26	1,024		128
189		8,192	256
68	1,024	1,024	2
62	256		32
63	4,096		64
61	1,024		
193		16,384	128
67	4,096	4,096	2,048
59	4,096		2,048
65	1,024		64
23	1,024		16
94		1,024	256
92		2,048	2
6		1,024	

RESULTS

Erwinia carotovora strains selected for antiserum production caused decay of potato tuber slices and formed typical colonies with deep depressions on CVP medium. Strains designated as *Eca* did not grow at 37 C, produced acid from α-methyl-D-glucoside, produced reducing substances from sucrose, and were phosphatase negative. Strains designated as *Ecc* formed colonies 1–2 mm in diameter in 48 hr at 37 C, did not produce acid from α-methyl-D-glucoside or reducing substances from sucrose, and also were phosphatase negative.

Agglutination titers of antisera produced with either untreated or fixed cells varied from 1:256 to 1:16,384 but 20 of 22 antisera had a titer of 1:1,024 or above (Table 1). In contrast, 12 of 15 antisera produced with heat-treated cells had titers of 1:256 or less. There were no apparent differences in titers when intramuscular or intravenous injections were used alone or in combination. In

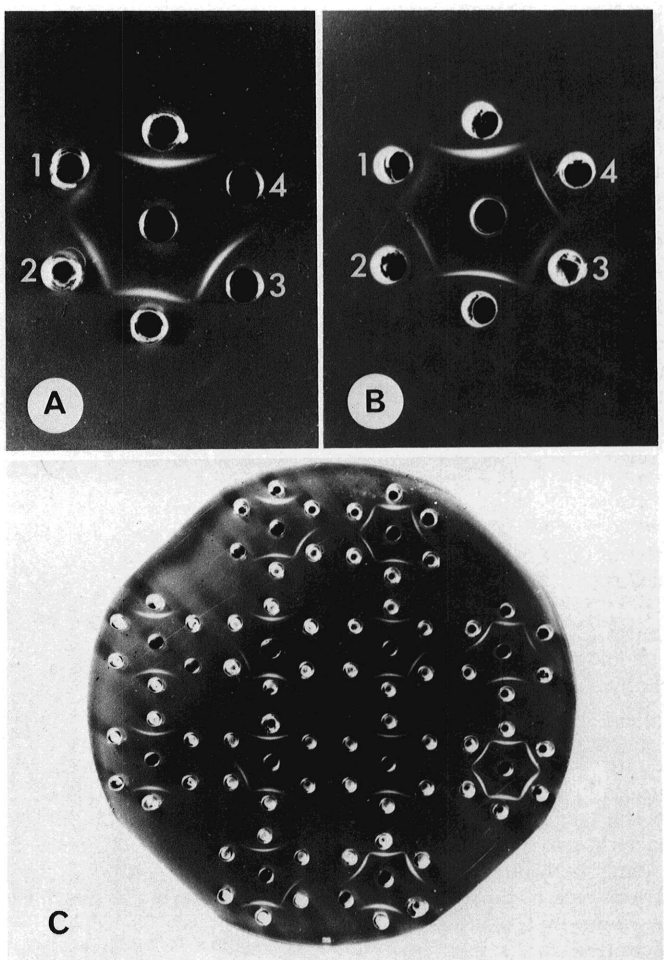

Fig. 1. Double diffusion patterns depicting reaction of serogroup I antiserum (center wells) in comparison with phenol-treated cells of the homologous strain (top and bottom wells of each set of six peripheral wells) with **A)** untreated homologous cells (well 1), heat-treated homologous cells (well 2), and a phenol-water extract from homologous cells (well 3), well 4 is empty; **B)** with phenol-treated cells of serogroup XVIII (wells 1 and 3) and II (wells 2 and 4); and **C)** with phenol-treated cells of unknown strains some of which were identified as serogroup I strains on the basis of their reaction of identity.

double diffusion, most antisera with titers above 1:256 gave a single, well-defined precipitin band, sometimes accompanied by additional weak bands with their homologous strains. Although precipitin bands were obtained with untreated cell suspensions, more distinct bands were obtained by the addition of phenol to the bacterial suspensions (Fig. 1A). Heat-treated suspensions of bacteria produced precipitin bands similar to those with non-heated cells (Fig. 1A). Phenol extracts prepared from strains 6, 24, 26, and 31 gave precipitin bands that fused with bands from untreated or phenol-treated cell suspensions, but an extract from isolate 63 did not react in double diffusion.

All strains were tested with the different antisera and if a precipitin band developed and fused with that of the homologous strain without spur formation to give a reaction of identity, the strains were considered to belong to the same serogroup (Fig. 1C). If a spur developed, or if there was no reaction, the strain was considered to be of another serogroup. On this basis 18 serogroups of potato strains were identified and designated by Roman numerals (Table 2). Serogroups I and XVIII were related as shown by a reaction of partial identity between strains; serogroup I, but not XVIII, was related to serogroup II (Fig. 1B). Other weak reactions of partial identity were observed among several other serogroups.

Of 1,001 potato strains from Canada, United States, Netherlands, and Germany that were tested, 829 (83%) could be placed into one of the 18 serogroups (Table 3). Although some serogroups occurred frequently, others were detected only once. The most common serogroups among potato strains isolated in British Columbia were I, III, IX, XI, and XVI (Table 4).

Some of the strains in serogroup III were difficult to classify since antisera produced against strain 24 gave two or three diffuse precipitin bands and the intensity of the different bands varied among strains. However, the presence or absence of spurs could be ascertained by viewing the double diffusion plates stereomicroscopically at ×10.

All 457 serogroup I strains tested were identified as *Eca* and two of 14 serogroup XVIII strains that were not *Eca* conformed to the characteristics of *Ecc* except one of these which did not grow at 37 C. All isolates in other serogroups were *Ecc*.

DISCUSSION

A high death-rate has been reported for rabbits immunized with *Erwinia* spp. (17) but no rabbits were lost due to toxic effects in our work. Differences in titers among antisera produced with untreated and glutaraldehyde-fixed cells probably represent variation in rabbit sensitivity rather than differences in antigenicity of the bacterial strains. The drop agglutination titer did not always reflect the intensity of the precipitin band produced by immunodiffusion, but antisera with titers of 1:256 or greater usually produced adequate precipitin bands for determining serological groups.

Because the Ouchterlony double diffusion technique permits identification of antigens in a mixture and can establish relationships between antigens, it can be used to arrange strains of bacteria into serogroups without complete knowledge of all the antigenic groups that may be present. In contrast, the Kauffmann-White scheme for serotyping *Salmonella* and other related genera is based on agglutination reaction with antisera rendered specific by absorption with cross-reacting antigens (16). Such a scheme, however, requires good knowledge of all antigenic determinants on the bacterial cell wall.

Enterobacteria have several surface antigens, including O, H, K, M, and Vi (8), but Kauffmann (16) found the LPS "O" antigen the most useful for primary serological grouping. The O antigen also has been used to group some strains of *E. chrysanthemi* (27). The antigens responsible for precipitin band formation in our work were not positively identified but probably are equivalent to the enteric O antigen (19) because they were heat stable and could be extracted from several strains of different serogroups with the phenol-water extraction procedure. Moreover, LPS similar in amount, physical appearance, and behavior to those from other enterobacteria have been purified from several *Erwinia* spp. (20,28).

Some enteric O antigens consist of several different O-factors which are common to more than one serogroup (8). O-factor analysis has not been done with *Ecc* or *Eca* but a relationship between serogroups, as shown by reactions of partial identity in double diffusion, indicates that they may share common O-factors.

The significance of secondary precipitin bands that may indicate the presence of other antigens has not been evaluated. The secondary bands sometimes constituted a problem in typing unknown strains in serogroup III when primary precipitin bands

TABLE 2. Strains of each *Erwinia carotovora* serogroup used to produce antisera

Serogroup	Strain[a] number	Variety	Original collector and his number
I	31	atroseptica	Kelman (SR8)
II	21	carotovora	Maas-Geesteranus (F,139)
III	24	carotovora	Maas-Geesteranus (163)
IV	190	carotovora	Kelman (SR204)
V	26	carotovora	Maas-Geesteranus (200)
VI	189	carotovora	Kelman (SR123a)
VII	68	carotovora	Kelman (SR165)
VIII	62	carotovora	Maas-Geesteranus (195)
IX	63	carotovora	Maas-Geesteranus (202)
X	61	carotovora	Maas-Geesteranus (222)
XI	193	carotovora	Copeman (E193)
XII	67	carotovora	Kelman (SR162)
XIII	59	carotovora	Maas-Geesteranus (257)
XIV	65	carotovora	Maas-Geesteranus (196)
XV	23	carotovora	Maas-Geesteranus (162)
XVI	94	carotovora	Copeman (E315)
XVII	92	carotovora	Copeman (E6)
XVIII	6	atroseptica	Copeman (E17)

[a]Strains listed here have been deposited in the International Collection of Phytopathogenic Bacteria, University of California, Davis, 95616.

TABLE 3. Number of *Erwinia carotovora* potato strains from several culture collections which could be placed in one of 18 serogroups

Collector	Strain origin	Number of strains tested	Number of strains typed
Maas-Geesteranus	Netherlands	48	36
	Arizona (Stangehellini)	16	14
	Germany (Webb)	2	2
Kelman	Wisconsin	25	21
Copeman	British Columbia	901	751
De Boer	Wisconsin	9	5
Total		1,001	829

TABLE 4. Frequency of serogroups among *Erwinia carotovora* strains isolated from symptomless seed and table-stock potato tubers and from potato plants with seed-piece decay or blackleg symptoms in British Columbia, Canada

Serogroup[a]	Number of strains		
	Symptomless seed and table-stock tubers	Plants with seed-piece decay or blackleg	Total
I	110	328	438
III	79	17	96
IV	6	2	8
VI	3	0	3
IX	29	5	34
XI	45	0	45
XV	1	0	1
XVI	18	8	26
XVIII	6	4	10
Not typed	32	65	97

[a]Serogroups not listed were not found among strains isolated in this survey.

were not well defined and the formation of spurs was unclear. it would have been advantageous to produce O-specific antisera, but the heat treatment method usually used to denature other surface antigens (8) yielded only low-titered antisera as did heat-treated *Proteus rettgeri* cells (23). Although heat treatment did not appear to destroy the O antigen (Fig. 1A), the treatment may have caused it to dissociate into nonantigenic subunits.

Because our results (Fig. 1B) confirm the serological relationship between *Eca* and *Ecc* shown by Vruggink and Maas-Geesteranus (32) we propose a single number sequence to designate serogroups of both varieties. Such a sequence also will accommodate strains that are biochemically different but serologically identical (eg, serogroup XVIII) as well as biochemically intermediate strains.

The serological homogeneity of *Eca* strains suggested by the work of others (1,29,32) was confirmed by the fact that most *Eca* strains were of a single serogroup (serogroup I). Serologically distinct *Eca* potato strains, however, were discovered and classified in a second serogroup (serogroup XVIII). Sugarbeet strains identified as *Eca* also have been reported to be serologically different from potato strains (29) and two sugarbeet strains tested did not react with serogroup I or XVIII antisera. Two strains biochemically equivalent to *Ecc* gave a reaction of identity with antisera XVIII, suggesting some instability of either the serological or biochemical characteristics.

Certain *Ecc* serogroups (III, IX, XI, and XVI) were isolated frequently from potato in British Columbia while other serogroups were isolated rarely or not at all. In The Netherlands, detection of *E. carotovora* by the immunofluorescence technique (31) in ostensibly healthy potato tubers showed that III and XIII were the most commonly found serogroups followed by IX and II (H. Vruggink, *unpublished*). The distribution of serogroups on potato may be fortuitous but perhaps particular serogroups such as serogroup III have a selective advantage on potato.

Additional serogroups are being established to include untyped strains, and strain relationships are being investigated. The limited scheme, however, already has provided some interesting epidemiological data which will be published later.

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