Serological and Physiological Differentiation Among Isolates of Erwinia carotovora from Potato and Sugarbeet

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ABSTRACT

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Antisera produced against six isolates of *Erwinia carotovora* were effective in differentiating sugarbeet from potato isolates of *E. carotovora* var. *atroseptica*. Two distinct biotypes of the sugarbeet pathogen were serologically and physiologically identified. Sugarbeet isolates from Willcox, AZ reacted only with antisera produced against an isolate from Willcox, whereas isolates from Washington, California, and Chandler, AZ reacted only with antisera produced against an isolate from Chandler. Methyl-β-D-galactopyranoside, galacturonic acid, and melibiose were utilized by all sugarbeet isolates from Willcox and most of the potato isolates, whereas sugarbeet isolates from Washington, California, and Chandler, AZ were incapable of utilizing these sugars. All sugarbeet isolates tolerated 7-9% NaCl in their culture medium and grew at 39 C, whereas potato isolates tolerated only 5-7% NaCl and did not grow at 39 C. Most sugarbeet isolates, but none of the potato isolates, produced a bacteriocin-like substance against *E. carotovora* var. *carotovora* (isolate E107). All sugarbeet isolates were pathogenic, whereas potato isolates, with the exception of isolate OBI, were nonpathogenic on sugarbeets. Isolate OBI, although obtained from potato, was physiologically and serologically identified as a Chandler sugarbeet biotype.

Additional key words: bacteriocin, blackleg.

Differentiation among varieties of *E. carotovora* commonly is based on the biochemical and physiological criteria established by Graham (4) and Dye (2, 3). Based on these criteria, Ruppel et al. (6) showed that the soft-rot bacterium associated with root rot of sugarbeets in California (9) and Washington (6) was a homogenous pathovar of *E. carotovora* which possessed the physiological and biochemical characteristics of *E. carotovora* var. *atroseptica*, but which could be distinguished from authentic isolates of the latter by means of pathogenicity tests on sugarbeets.

Bacterial root rot of sugarbeets, previously reported only in Washington (6) and California (9), was observed for the first time in 1974 in Chandler and Willcox, Arizona. Pectolytic *Erwinia* spp. were consistently isolated from infected root tissue and identified, using biochemical and physiological tests (2, 3, 4, 7), as *E. carotovora* var. *atroseptica*. Preliminary investigations, initiated to determine the long-term survival potential of the sugarbeet bacterium in soil in the absence of a susceptible host crop, showed that isolates of *E. carotovora* var. *atroseptica*, both virulent and avirulent to sugarbeets, as well as other pectolytic *Erwinia* sp., could be recovered from the rhizosphere of weeds and soil from fields previously cropped to sugarbeets (5). Differentiation between these isolates would necessitate greenhouse pathogenicity tests on sugarbeets. Thus, the following investigation was initiated to: (i) find more rapid tests which would distinguish the sugarbeet pathogen from other isolates of *E. carotovora* var. *atroseptica*, and (ii) determine the physiological and serological characteristics of sugarbeet isolates from diverse geographical regions of the U.S.

MATERIALS AND METHODS

Sixty-four isolates of *E. carotovora* from diverse geographical regions were used (Table 1). Stock cultures of each isolate, unless otherwise specified, were maintained on yeast-dextrose-carbonate (YDC) agar slants (7).

Pathogenicity tests.—Pathogenicity of all isolates was determined using a modification of Ruppel's (6) method: 0.5 ml of a turbid cell suspension (about 10^7 cells/ml of sterile distilled water) from a 24-hr-old culture of each isolate was placed in the crown of a wounded 3-mo-old sugarbeet plant (US H9B) growing in a 15-cm diameter plastic pot containing a pasteurized mixture of equal volumes of sand, peat, and soil. Wounds were inflicted by puncturing the crown several times with a sterile dissecting needle. Inoculated and appropriate noninoculated control plants were incubated in a greenhouse (25-29 C) and inspected periodically over a 20-day period for crown rot and root decay as described by Ruppel et al. (6). Pathogenicity tests were repeated twice.

Serological tests.—Cultures used for antisera production were prepared, according to the method of Allan and Kelman (1, and A. Kelman, *personal*

communication), as follows: bacterial cells were harvested after 48 hr of growth at 27 C on a medium containing 0.1% casamino acids, 1% peptone, 1% glucose, and 1.8% agar. Harvested cells were suspended in phosphate buffered saline (PBS - 0.01 M K_2HPO_4-KH_2PO_4 buffer at pH 7.2; 0.85% NaCl) and washed three consecutive times in PBS by centrifugation for 15 min at 12,000 *g*. Washed cell suspensions were then dialyzed against 2% glutaraldehyde at 4 C for 3-4 hr followed by dialysis against PBS with frequent changes at 4 C for 20 hr. Washed cells, resuspended and adjusted to about 10^9 cells/ml in PBS, were used for immunization of rabbits.

One New Zealand white doe rabbit (3-4 kg) was immunized for each bacterial isolate. Normal sera were obtained prior to immunization. One ml of each bacterial antigen was emulsified with 1 ml of Freund's incomplete adjuvant (Difco) and injected intramuscularly into the hind leg of each rabbit. Injections were given weekly over a 7-wk period. Blood was collected by cardiac puncture from each rabbit at each of three bleedings initiated 45, 52, and 59 days after the first injection. The blood was stored at 4 C for 12 hr. After clotting, sera were collected

TABLE 1. Isolates of *Erwinia carotovora* used for comparative studies of pathogenicity, and serological and physiological characteristics

Varieties of *Erwinia carotovora* (*sensu* Graham)	Host	No. of isolates	Obtained from	Location	Isolate identification number
atroseptica	*Solanum tuberosum* L.	3	Kelman	Wisconsin	SR8, SR160, SR155
		2	Sands	Connecticut	C250, C249
		1	Harrison	Colorado	AS-1-2b2
		12	Stanghellini	Arizona	PF27, PT 2, 0'5, 35, NOBT, NOBK, N'8, PF18, OB103, PF8, PF15, OBI
carotovora	*Solanum tuberosum* L.	1	Kelman	Wisconsin	SR162
		25	Stanghellini	Arizona	NOBM, SF10, 58, 46, 67, OB103, OBD, SF1, 0'4, SF3, N'6, NOB102, FT6, 49, 47, 50, 63, 34, 66, 44, 2, 45, 25, 59, 65
atroseptica	*Beta vulgaris* L.	8	Stanghellini	Willcox, AZ	KSB 1 to 8
		6	Stanghellini	Chandler, AZ	CB 1 to 6
		2	Thomson	California	ShSB-1, UR-7
		4	Ruppel	Washington	DMSB-1, WE-1C1 MLSB-1, WE-1a1

TABLE 2. Results of pathogenicity and serological tests used to differentiate sugarbeet from potato isolates of *Erwinia carotovora*

Host	Variety of *E. carotovora* (*sensu* Graham)	Source	Isolates tested (no.)	Reaction with antisera[a] to isolates KSB6	CB2	OBI	PF18	PF27	50	Pathogenic on sugarbeet
Beta vulgaris	'atroseptica'	California	2	–[b]	+	+	–	–	–	+
		Washington	4	–	+	+	–	–	–	+
		Chandler, AZ	6	–	+	+	–	–	–	+
		Willcox, AZ	8	+	–	–	–	–	–	+
Solanum tuberosum L.	'atroseptica'	Wisconsin	3	–	–	–	+	+	–	–
		Connecticut	2	–	–	–	+	+	–	–
		Colorado	1	–	–	–	+	+	–	–
		Arizona	11	–	–	–	+	+	–	–
		Arizona (OBI)	1	–	+	+	–	–	–	+
Solanum tuberosum L.	'carotovora'	Wisconsin	1	–	–	–	–	–	–	–
		Arizona	17	–	–	–	–	–	–	–
		Arizona	7	–	–	–	–	–	+	–
		Arizona	1	–	–	–	–	+	+	–

[a]Isolate KSB6 and CB2 are sugarbeet isolates from Willcox and Chandler, Arizona, respectively. Isolate OBI, PF18, and PF27 are potato isolates of *E. carotovora* var. *atroseptica* from Arizona. Isolate 50 is a potato isolate of *E. carotovora* var. *carotovora* from Arizona.

[b]Reactions visually rated as positive (+) (agglutination) or negative (–) (no agglutination detected).

TABLE 3. Physiological tests used to differentiate sugarbeet from potato isolates of *Erwinia carotovora* var. *atroseptica*

Host species	Source	Isolate identification no.	Methyl-β-D-galactopyranoside utilization	Galacturonic acid utilization	Melibiose utilization	Growth at 39 C	Maximum % NaCl supporting growth	Bacteriocin-like activity against isolate E107[a]
Solanum tuberosum	Arizona	OBI	–[b]	–	–	+	8	+
		pF27	+	+	+	–	5	–
		0'5	+	+	+	–	5	–
	Connecticut	C249	–	+	+	–	6	–
		C250	+	+	±	+	7	–
	Wisconsin	SR-8	+	+	+	–	6	–
		SR160	+	+	+	–	6	–
		SR155	–	+	+	–	5	–
	Colorado	AS-12B2	–	+	+	–	5	–
Beta vulgaris	California	UR-7	–	–	–	+	8	+
		SHSB-1	–	–	–	+	8	+
	Washington	DMSB-1	–	–	–	+	8	–
		MLSB-1	–	–	–	+	8	–
		WE1C1	–	–	–	+	8	+
		WE1a1	–	–	–	+	8	–
	Chandler, AZ	CB_2-1	–	–	–	+	7	+
		CB_{2-2}	–	–	–	+	7	+
		CB_{2-3}	–	–	–	+	7	+
		CB_2-4	–	–	–	+	7	+
	Willcox, AZ	KSB-1	+	+	+	+	8	+
		KSB-2	+	+	+	+	8	+
		KSB-3	+	+	+	+	8	+
		KSB-4	+	+	+	+	9	±
		KSB-5	+	+	+	+	9	+
		KSB-6	+	+	+	+	8	+
		KSB-7	+	+	+	+	8	+
		KSB-8	+	+	+	+	8	+

[a]Bacteriocin-like activity rated as positive (+); negative (–); or variable (±). Isolate E 107 was identified as *E. carotovora* var. *carotovora* (sensu Graham).

[b]Sugar utilization rated as (+) = growth; (–) = no growth; or (±) = slight growth.

by decanting and stored at −20 C until used.

Titers of antisera were determined by slide agglutination tests using PBS washed whole bacterial cells (about 10^9 cells/ml) obtained from 2-day-old YDC agar slant cultures. Antiserum dilutions from 2 to 30,000 were prepared with physiological saline. Titers obtained ranged between 10,000 and 20,000. For heterologous cross agglutination tests, 1 ml of a 1:20 dilution of each antiserum was mixed with 0.1 ml of each of the bacterial suspensions and incubated at 28 C. Reactions were visually recorded as positive (agglutination) or negative (no detectable agglutination). All tests were repeated at least twice over a 2 mo period.

Physiological tests.—Representative isolates of *E. carotovora* var. *atroseptica* from potato and sugarbeet were selected for physiological tests. Isolates of *E. carotovora* var. *carotovora* were not tested since existing biochemical and physiological tests (2, 3, 4) were adequate to differentiate between varieties of *E. carotovora*. Utilization of methyl-β-D galactopyranoside (Sigma Chemical Co., St. Louis, MO 63178), galacturonic acid, and melibiose was determined by growth on a mineral salts (10), 1.1% Noble agar (Difco) medium. Stock sugar solutions, adjusted to neutral pH and filter-sterilized, were added after autoclaving the basal medium to give a final sugar concentration of 0.1%. Growth at 30 C in agar plates without added sugars was compared with growth in plates with sugars after 2 and 4 days. Tolerance to sodium chloride and growth at 39 C were determined by the methods of Dye (2). Bacteriocin-like activity was assayed on a medium containing Bacto tryptone, 10 g; yeast extract (Difco), 5 g; NaCl, 5 g; and agar (Difco), 15 g per liter of glass-distilled water (TY-agar). Bacterial cultures were spotted onto the agar and incubated overnight at 30 C. The petri dishes were inverted over chloroform for 3-5 min to kill the bacteria, aerated for 30 min, and flooded with 5 ml of soft TY-agar (0.7% agar) seeded with 10^5 cells/ml of the test bacterium (*E. carotovora* var. *carotovora* isolate E-107) grown overnight in TY-broth. Plates were observed the next day for zones of inhibition. Zones greater than 2 mm beyond the margin of killed colonies were recorded as positive. All physiological tests were performed at least twice.

RESULTS

Pathogenicity tests.—Disease symptoms, typical of those reported by Ruppel et al. (6), developed on all sugarbeets inoculated with the sugarbeet isolates. Black lesions on petiole bases and production of foam in the crown was observed within 5 to 8 days and internal decay of the tap root was evident within 15 days after inoculation. Potato isolates, with a single exception (isolate OBI), were nonpathogenic on sugarbeets (Table 2).

Serological tests.—Potato isolates of *E. carotovora* var. *atroseptica*, with the exception of isolate OBI, were serologically identical and could be differentiated from both sugarbeet isolates of *E. carotovora* var. *atroseptica* and potato isolates of *E. carotovora* var. *carotovora* (Table 2). Isolate OBI, the only potato isolate among 46 tested that was pathogenic on sugarbeets, was also the only potato isolate that was serologically related to a sugarbeet isolate (isolate CB_2).

Sugarbeet isolates, although serologically distinct from potato isolates, were not homogenous. Isolates from California, Washington, and Chandler, Arizona, were serologically identical, but were not related serologically to sugarbeet isolates from Willcox, Arizona.

Physiological tests.—Physiological tests that appear to be of differential value are listed in Table 3. All sugarbeet isolates grew at 39 C and tolerated 7% NaCl or more in their culture medium, whereas only two of nine potato isolates could do so. Isolate OBI, one of the two potato isolates, was capable of infecting sugarbeets and was physiologically similar to all the sugarbeet isolates from California, Washington, and Chandler, Arizona. The latter isolates were incapable of utilizing methyl-β-D-galactopyranoside, galacturonic acid, or melibiose. All sugarbeet isolates from Willcox, Arizona, were capable of utilizing these sugars. Most of the sugarbeet isolates, but none of the potato isolates, produced a bacteriocin-like substance against isolate E107 of *E. carotovora* var. *carotovora*.

DISCUSSION

Disease control is frequently dependent upon knowledge of the ecology, epidemiology, and genetic variability of the pathogen. Prerequisite for control is the accurate identification and/or recognition of the pathogen. Results of this investigation indicate that the soft rot bacterium associated with root rot of mature sugarbeets can be differentiated from potato isolates of *E. carotovora* var. *atroseptica* and var. *carotovora* by means of serological, physiological, and [as demonstrated by Ruppel et al. (6)] pathogenicity tests. Additionally, accurate identification through serological and physiological tests indicates that the sugarbeet pathogen, although an apparent pathovar of *E. carotovora* var. *atroseptica*, is not homogenous. Distinct serological and physiological biotypes of the sugarbeet pathogen were found in widely separated sugarbeet production areas. The pathogenic diversity of these biotypes is not known but might be taken into consideration in any program aimed at breeding for resistance.

Ruppel et al. (6) and Thomson et al. (9) demonstrated that the sugarbeet pathogen, in greenhouse inoculation studies, would cause blackleg of potato. Identification of one of the sugarbeet biotypes among potato isolates of *E. carotovora* var. *atroseptica* demonstrates that the sugarbeet pathogen is not restricted in nature to sugarbeets. This finding also strengthens previous arguments (7) that more than one variety or biotype of *E. carotovora* is or can be associated with blackleg of potato.

The origin of the sugarbeet pathogen is not known. The occurrence of the disease in diverse geographical areas coincided with the widespread use of virus yellows-resistant sugarbeet cultivars (US H9 and US H10) in 1971 (9). Though it is possible that the pathogen may have been disseminated on infested or infected sugarbeet seed, attempts to demonstrate the seed-borne nature of the pathogen (8, and M. E. Stanghellini, *unpublished data*) have not been successful. The existence, however, of more than one biotype may indicate regional selection of a specific biotype of the pathogen by the host from an existing heterogenous population (5) residing naturally in soil. Studies on the soil-borne nature of soft-rot *Erwinia* spp. are currently under investigation.

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