Pectobacterium spp. Associated with Bacterial Stem Rot Syndrome of Potato in Canada

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ABSTRACT

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Pectobacterium atrosepticum, *P. carotovorum* subsp. *brasiliensis*, *P. carotovorum* subsp. *carotovorum*, and *P. wasabiae* were detected in potato stems with blackleg symptoms using species- and subspecies-specific polymerase chain reaction (PCR). The tests included a new assay for *P. wasabiae* based on the phytase gene sequence. Identification of isolates from diseased stems by biochemical or physiological characterization, PCR, and multi-locus sequence typing (MLST) largely confirmed the PCR detection of *Pectobacterium* spp. in stem samples. *P. atrosepticum* was most commonly present but was the sole *Pectobacterium* sp. detected in only 52% of the diseased stems. *P. wasabiae* was most frequently present in combination with *P. atrosepticum* and was the sole *Pectobacterium* sp. detected in 13% of diseased stems. Pathogenicity of *P. wasabiae* on potato and its capacity to cause blackleg disease were demonstrated by stem inoculation and its isolation as the sole *Pectobacterium* sp. from field-grown diseased plants produced from inoculated seed tubers. Incidence of *P. carotovorum* subsp. *brasiliensis* was low in diseased stems, and the ability of Canadian strains to cause blackleg in plants grown from inoculated tubers was not confirmed. Canadian isolates of *P. carotovorum* subsp. *brasiliensis* differed from Brazilian isolates in diagnostic biochemical tests but conformed to the subspecies in PCR specificity and typing by MLST.

Blackleg is a common and ubiquitous bacterial disease of potato. It is characterized by symptoms of decay and black discoloration of affected tissue that develops at the base of potato stems. Early in the growing season, underground stem decay results in stunted plants with stiff and yellowing foliage, which succumb within weeks of emergence, whereas blackleg that develops during midseason and thereafter manifests itself as decaying, black-colored stems with wilting or yellowing leaves. Affected stems usually become entirely decayed prior to crop maturity, dry out, and are obscured by the foliage canopy. Progeny tubers produced on diseased stems sometimes but not always become infected via the stolon and decay during the growing season or after harvest in storage. Lenticellular infection of progeny tubers also occurs from inoculum that spreads from decaying seed tubers into the root zones of affected plants.

In North America and Western Europe, the main causal agent of potato blackleg has been *Pectobacterium atrosepticum* (syn. *Erwinia carotovora* subsp. *atroseptica*), a gram-negative, motile bacterium physiologically and genomically similar to enteric bacteria associated with food spoilage and mammalian diseases but differing from them significantly in growth temperature optima and host preference. Although sometimes considered an opportunistic pathogen, *P. atrosepticum* has an intricate repertoire of genomic traits that equip it in a very special way for its coexistence and virulence on its host crop plant (9). Potato strains of *P. atrosepticum* exhibit a very limited degree of diversity, with the majority of strains typing into a single clade whether analysis is based on serology (10), biochemical tests (47), phage specificity (54), or molecular assays (54,60).

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P. atrosepticum has also been implicated in diseases of various other crops besides potato such a tomato (32), pepper (50), sunflower (2,17), and tobacco (56). However, like the sugar beet pathogen, which was initially classified as *E. carotovora* subsp. *atroseptica* based on limited biochemical characterization (49), more extensive study revealed that, with some exceptions, non-potato isolates differ from the potato blackleg pathogen (12). The decay-causing sugar beet strains were ultimately reclassified as *P. betavasculorum* (18,52) but the taxonomic position of some of the other *P. atrosepticum*-like strains remains ambiguous.

Several reports from the United States and elsewhere suggested that *P. carotovorum* can also incite the blackleg disease, particularly under conditions of high field temperature (33,34,43,48,51); and, recently, de Haan et al. (14) provided solid evidence, based on Koch's postulates, that certain strains of *P. carotovorum* indeed incite symptoms of blackleg in potato stems grown from inoculated seed tubers. Generally, however, most strains of *P. carotovorum* do not cause blackleg but are able to infect potato stems through wounds caused by insects, fungal infection, or farm implements during cultural maintenance of the crop. The symptoms of such stem infections, also known as aerial soft rot, are very different from those of blackleg in that they may turn various shades of brown but do not become inky black, as is common for the blackleg disease. A characteristic of the blackleg disease that differentiates it from aerial stem rot is that it originates belowground at the seed tuber and moves upward into the stem. Even though symptoms of blackleg may first break out at an aboveground node in the stem, infections can always be traced back through the vascular tissue or pith to the belowground portion of the stem, where the infection originated at the seed tuber. Aerial stem rot does not originate from the seed tuber although, in stems with advanced decay, differentiation between blackleg and aerial stem rot often becomes blurred.

Rather surprisingly, it was discovered recently that the potato blackleg disease in Brazil was not caused by *P. atrosepticum* but by a new subspecific clade within *P. carotovorum* which was

tentatively named *E. carotovora* subsp. *brasiliensis* (currently *P. carotovorum* subsp. *brasiliensis*) (15). In fact, *P. carotovorum* subsp. *brasiliensis* appears to be the only causal agent of potato blackleg in Brazil because *P. atrosepticum* was not detected in an extensive survey of potato *Pectobacterium* spp. in the Brazilian state of Rio Grande do Sul (16). More recently, *P. carotovorum* subsp. *brasiliensis* was also identified as the major cause of potato blackleg in South Africa, where it has become a disease of significant economic importance (59). Strains of *P. carotovorum* subsp. *brasiliensis* grow at higher temperatures and are more virulent than *P. atrosepticum* strains (15), and clustered closer to *P. carotovorum* than *P. atrosepticum* in analyses based on biochemical characteristics (15) and housekeeping gene sequences (31). Because of its phylogenetic distinctiveness, it has been suggested that separate species status for *P. carotovorum* subsp. *brasiliensis* is warranted (19).

Another species of *Pectobacterium*, *P. wasabiae*, first described as causing soft rot of Japanese horseradish (23), was identified among *Pectobacterium* spp. isolated from decayed potato tubers in New Zealand (39,40). These isolates not only caused tuber decay but also incited blackleg-like symptoms in stem-inoculated potato plants (40). Similarly, *P. wasabiae* was isolated from decayed potato tubers in the United States (Wisconsin) and Iran and similarly caused stem decay in potato plants inoculated for a virulence assay (1,26). Although both the Wisconsin and Iranian strains of *P. wasabiae* caused decay of potato stem and tuber tissues upon inoculation, ability to cause the blackleg disease naturally by invasion of stem tissue from the seed piece has not yet been demonstrated.

In the early 1970s, H. P. Maas Geesteranus found that potato stem rot in the Netherlands was sometimes caused by an unusual *Erwinia*-like bacterium that was ultimately identified as a *Dickeya* sp. (syn. *E. chrysanthemi* (H. P. Maas Geesteranus, *personal communication*). Although symptoms of this disease, known as "stengelnatrot" (stem wet rot) in the Netherlands, resembles potato blackleg, pith decay is more pronounced than in *P. atrosepticum*-caused blackleg during the early stages of the disease, and externally visible stem decay is less prevalent. At about the same time that stengelnatrot was recognized as a new disease in the Netherlands, a *Dickeya* sp. was also identified as the causal agent of a blackleg disease of potato in the Niigata prefecture of Japan (53) and tuber soft rot in the semiarid irrigated sands of southwestern New South Wales, Australia (3). Subsequently, the association of *Dickeya* spp. with potato disease was reported from France (41), Israel (30), and South Africa (43). Recently, *Dickeya* sp.-caused blackleg has become a major disease problem throughout Europe, including the Netherlands (6), France (24), Spain (36), Finland (29), and Poland (45), as well as in Israel, which imports seed potato from Europe (57,58). The various biotypes of the previous *E. chrysanthemi* species have now been distributed among six *Dickeya* spp. but each of the species has been associated with potato disease. A newly described strain of *Dickeya* is of increasing economic importance as a cause of potato disease and, perhaps, warrants separate species status (29,46). The name *Dickeya solani* has been proposed (55).

Anticipated reduction in tuber-borne bacterial soft rot diseases with the introduction of pathogen-tested stem cuttings and aseptic tissue cultures for virus elimination in seed potato programs has only been partially realized (21,62). Although some reduction in disease occurred (8,20,22), blackleg continues to plague the industry and, according to observations by industry stakeholders and anecdotal accounts, is actually increasing again in some regions of Europe and North America. These observations were the impetus to reevaluate the etiology of blackleg and stem rot of potato in Canada using molecular diagnostic tools not previously available. The objective of this study was to ascertain whether or not *P. atrosepticum* was still the primary cause of blackleg, as has been the case, or whether the potato industry was faced with a changing scenario in which different *Pectobacterium* spp. contributed to a more complex bacterial stem rot syndrome.

MATERIALS AND METHODS

Bacterial strains, media, isolation, maintenance, and characterization. Reference strains for the different *Pectobacterium* taxa used in this study were as follows: *P. atrosepticum* strain 3 and 31 (11); *P. carotovorum* subsp. *carotovorum* strain 26 and 71 (11); *P. carotovorum* subsp. *brasiliensis* strains 8, 212, and 371 (15); and *P. wasabiae* SR94 (23). These strains were from the laboratory collection maintained in 20% glycerol at –80°C and were grown routinely on nutrient agar. New isolates of *Pectobacterium* spp. were obtained from diseased potato stems and tubers by selecting colonies that developed pits in crystal violet pectate (CVP) medium (4) and visually resembled *Pectobacterium* colony morphology when viewed with a dissecting microscope using oblique illumination. Colonies picked from CVP medium were plated on casamino acid-peptone-glucose or nutrient agar medium to obtain pure cultures, and were maintained in 20% glycerol at –20°C. Biochemical or physiological tests to differentiate *Pectobacterium* isolates, including ability to grow at 36°C, production of acid from α-methylglucoside, production of reducing substances from sucrose, and phosphatase activity, were routinely carried out on fresh cultures using reference strains as controls according to standard published procedures (42).

Potato samples. Samples of potato stems expressing blackleg-like symptoms and associated tubers with decay were sent to the laboratory by various collaborators in the Canadian provinces of Alberta, Saskatchewan, Manitoba, and Prince Edward Island. Upon receipt, samples were processed immediately or kept at –20°C until processed. For each sample, 0.5 to 1.0 g of diseased tissue, where possible at the interface with healthy tissue, was macerated in 1 ml of distilled water in an extraction bag (BioReba, Reinach, Switzerland). Liquid from the bag (1 ml) was used for extracting DNA (referred to as sample DNA hereafter) using a KingFisher magnetic particle processor (Thermo Fisher Scientific Inc., Toronto). For some of the samples, a separate aliquot was directly plated on CVP medium, or plated after incubation for 3 to 4 days in enrichment broth at room temperature (42), for isolation of *Pectobacterium* spp. Symptomatic stems from field trials were sampled in the same way, whereas asymptomatic tubers from field trials were tested by removing 0.5 g of tissue from the stolon end and macerating the tissue in extraction bags using a Homex 6 homogenizer (BioReba).

Polymerase chain reaction of sample DNA. Conventional polymerase chain reaction (PCR) using taxon-specific primers (Table 1) was used to determine which *Pectobacterium* spp. or subspecies were present in individual plant samples. PCR was performed with Sprint Advantage Single Shot (Clontech, Mountain View, CA) reagents and primer concentrations of 0.5 μM. After denaturation for 5 min at 95°C, 40 cycles were run at 95°C for 30 s, 62°C for 30 s (except 60°C for primer set PhF/PhR), and 72°C for 30 s, followed by a final extension at 72°C for 4 min. PCR products were analyzed by capillary gel electrophoresis (Qiagen, Mississauga, ON, Canada) or electrophoresis in 1% agarose gels.

Development of a PCR assay for *P. wasabiae*. A PCR assay was developed for *P. wasabiae* based on the GenBank sequence (EU203663) of the novel phytase gene, *appA*, that had been cloned and sequenced from the type strain (DSMZ18074 = SR91) (44). Primers were selected using Allelle ID software (Premier Biosoft, Palo Alta, CA). Specificity of the PCR assay was based on the failure to obtain amplification products from known non-*P. wasabiae* isolates of *Pectobacterium* and uniqueness of the DNA sequence determined in BLAST analysis of the GenBank nucleotide database. A preliminary report has been published (61).

PCR and multi-locus sequence typing identification of isolates. Pure cultures of isolates provisionally identified by biochemical and physiological tests were further characterized by PCR using taxon-specific primers as indicated above for sample DNA. Additionally, isolates were characterized by multi-locus sequence typing (MLST) targeting either two or six housekeeping genes (*acnA*, *gapA*, *icdA*, *mdh*, *pgi*, and *proA*), as described by Ma et al. (31). When all six genes were included, both concatenated and individual sequences were aligned and analyzed using AlignPlus 4 (version 4.1) of Clone Manager Professional Suite with default settings along with a collection of comparable sequences of Ma et al. (31) retrieved from GenBank. Routinely, identity of isolates was confirmed by sequencing only the *acnA* and *proA* gene fragments and determining their phylogenetic affinities based on the associated clade when compared and analyzed with a collection of homologous GenBank sequences.

Pathogenicity testing. Maceration ability of isolates was determined in both green pepper fruit and potato tubers. Green pepper fruit from a local grocer were surface sterilized with 1% commercial bleach and 70% ethanol, thoroughly rinsed before being cut longitudinally, and inoculated with *Pectobacterium* isolates using a toothpick dipped into a colony of a 48-h-old bacterial culture. The diameter of decay surrounding the inoculation point was measured 48 h after incubation of inoculated pepper at 23°C. Potato tubers were similarly surface sterilized and inoculated with *Pectobacterium* spp. using the same toothpick method, and covered with a thin film of mineral oil. After 72 h, tubers were cut through the middle of inoculation sites and the diameter of decay was measured at the widest point.

To determine stem-rotting ability, stems of 8-week-old greenhouse-grown potato plants (two each of 'Fabula', 'Norland', 'Superior', and 'Yukon Gold') were inoculated at lower leaf axils with toothpicks dipped into bacterial colonies. Plants were inoculated individually with five isolates of *P. atrosepticum*, six isolates of *P. carotovorum* subsp. *brasiliensis*, and four isolates each of *P. carotovorum* subsp. *carotovorum* and *P. wasabiae*. Symptoms of decay and leaf wilting were rated separately on a scale of 0 to 5 (0 = no symptoms and 5 = decay causing stem collapse or wilt of all leaves) over a 12-day period. Disease index was calculated as the sum of the decay and wilt ratings. The stem inoculation experiment was repeated on a set of six greenhouse-grown plants (Superior) per *Pectobacterium* taxon.

Field trials. Ability of isolates of the different *Pectobacterium* taxa to cause blackleg-like disease was tested in field plots using inoculated seed tubers to simulate a natural pathway of infection. Surface sterilized, high-class (Elite I) seed potato tubers (Superior) were vacuum infiltrated with suspensions of bacterial isolates obtained in this study, diluted 1/1000 in water from suspensions adjusted to an optical density at 600 nm of 1.0, and air dried. In 2010, tubers were inoculated 1 week before planting. At planting, tubers were cut into seed pieces and immediately planted by treatment in six rows 1 m apart. Each treatment (consisting of 20 seed tubers/subplot) was situated randomly within the plot and flanked within rows by unplanted 5-m spaces. The *P. atrosepticum* (three isolates), *P. wasabiae* (three isolates), *P. carotovorum* subsp. *brasiliensis* (two isolates), and *P. carotovorum* subsp. *carotovorum* (two isolates) treatments as well as a water control treatment were replicated five times within the plot. The same plot layout was duplicated in an irrigated and an unirrigated field. In the irrigated field, irrigation was applied to supplement rainfall as required to maintain high soil moisture at all times. Cultivation, fertilization, and pesticide applications conformed to local commercial potato-growing practices.

In 2011, a similar field plot was established with potato tubers (Superior) inoculated the same way as in 2010 but planted 3 weeks after inoculation. Plot layout was similar to that used in 2010 except that all replications of each treatment were in adjacent rows, separated from other treatments by control water-inoculated subplots, and flanked within rows by unplanted 5-m spaces. Each of two strains of each species or subspecies were considered a treatment and replicated in five subplots within the plot. As in 2010, duplicate plots were planted for an irrigated and an unirrigated trial; plots were again managed in conformance to local commercial practices.

In both the 2010 and 2011 field trials, incidence of blackleg disease was monitored weekly and a few symptomatic plants were collected during the growing season for testing by PCR using taxon-specific primers. Isolations were made from some of the diseased stems in 2011. Plants were chemically top killed 97 and 91 days after planting in 2010 and 2011, respectively, and harvested 19 and 13 days after top-kill application, respectively. At harvest, tubers were taken randomly from some of the plots to test for latent infections with *Pectobacterium* spp. Tubers were tested by processing 0.5-g tissue samples from the stolon ends as described above and tested individually or combined into composites of 5 or 10 samples before testing by PCR using taxon-specific primers.

RESULTS

PCR detection of *Pectobacterium* spp. in blackleg-infected potato stems. Samples of potato stems with blackleg symptoms were received from 30 different commercial potato fields in 2007 to 2009 (Table 2). *P. atrosepticum* was detected by PCR in samples from 90% of the fields. *P. wasabiae* and *P. carotovorum* subsp. *brasiliensis* were also detected in 37 and 7% of the fields, respectively. Although the PCR for *P. carotovorum* subsp. *carotovorum* was also positive for 23% of the fields, the primers used had poor specificity for the subspecies, amplifying some strains of *P. wasabiae* (25) and not amplifying all strains of *P. carotovorum* subsp. *carotovorum*.

P. atrosepticum was detected in 86% of the individual stems with blackleg symptoms (Table 2) but was the only *Pectobacterium* sp. detected in 52% of them. *P. wasabiae* was the second most common *Pectobacterium* sp. detected, being found in 36% of symptomatic stems, and the sole *Pectobacterium* sp. detected

TABLE 1. Nucleotide sequence of taxon-specific primers used in this study

Taxon	Gene target	Primer name	Primer sequence	Reference
Pectobacterium atrosepticum	Genome	ECA1f	CGG CAT CAT AAA AAC ACG	
		ECA1r	GCA CAC TTC ATC CAG CGA	13
P. carotovorum subsp. *brasiliensis*	Intergenic spacer of rrn operon	BR1f	GCG TGC CGG GTT TAT GCA CT	
		L1r	CAA GGC ATC CAC CGT	15
P. carotovorum subsp. *carotovorum*[a]	Genome	EXPCCF	GAA CTT CGC ACC GCC GAC CTT CTA	
		EXPCCR	GCC GTA ATT GCC TAC CTG CTT AAG	25
P. wasabiae	Phytase gene	PhF	GGTTCAGTGCGTCAGGAGAG	
		PhR	GCGGAGAGGAAGCGGTGAAG	This study
Dickeya sp.	pel gene	ADE1	GAT CAG AAA GCC CGC AGC CAG AT	
		ADE2	CTG TGG CCG ATC AGG ATG GTT TTG TCG TGC	35

[a] Known to also amplify some *P. wasabiae* strains (25).

in 13% of individual stems. *P. carotovorum* subsp. *brasiliensis* and *P. carotovorum* subsp. *carotovorum* were detected at lower incidences (Table 2) and never as the sole *Pectobacterium* sp. present.

Isolation of *Pectobacterium* spp. from blackleg-infected stems was attempted and successful for only some of the stems from the commercial field sources. In every case, the species or subspecies of *Pectobacterium* isolated had been detected by PCR in sample DNA, with only one exception, in which *P. carotovorum* subsp. *brasiliensis* was isolated but had not been detected by PCR.

Isolation and characterization of *Pectobacterium* isolates. Isolation from diseased potato stems yielded 30 isolates. An additional six isolates were obtained from decayed potato tubers submitted separately to the laboratory. Good correlation was obtained between the different methods used to identify the isolates, which included biochemical characterization, PCR using taxon-specific primers, and MLST (Table 3). Two pectin-degrading isolates having phosphatase activity and whose DNA was amplified in PCR using the *Dickeya* spp.-specific primers were presumptively identified as *Dickeya* spp. and not tested further in this study. Consistent with the frequency at which *P. atrosepticum* was detected by PCR in diseased stems, most of the isolates (63%) were identified as *P. atrosepticum* by their biochemical and physiological characteristics (Table 3). *P. wasabiae* strains were differentiated from *P. carotovorum* strains by their inability to grow at 36°C and their identities confirmed by results of the taxon-specific PCR and MLST. The isolates identified as *P. carotovorum* subsp. *brasiliensis* reacted as *P. carotovorum* subsp. *carotovorum* strains in the differential biochemical tests in contrast to the control Brazilian strain (Table 3). However, these isolates were deemed to be *P. carotovorum* subsp. *brasiliensis* on the basis of DNA amplification in the taxon-specific PCR and MLST.

Initial identification using MLST was by the method of Ma et al. (31), in which sequences of six gene fragments were concatenated and sorted into clades within a dendrogram. Grouping of unknown strains into the clades identified by Ma et al. (31) was taken as evidence of isolate identity. Housekeeping gene sequences were also analyzed separately and found, with few exceptions, to group strains in the same way as the concatenated gene sequences. Hence, two housekeeping genes, *proA* and *acnA*, were selected for routine sequencing and analysis to rapidly identify new isolates (Fig. 1). Some strains of each *Pectobacterium* taxon were identified by MLST using concatenated sequences but, subsequently, new isolates were identified by analysis of only the two genes.

Identification of older *Pectobacterium* isolates in a culture collection. To determine whether the recent detection of *P. carotovorum* subsp. *brasiliensis* and *P. wasabiae* on potato is a new phenomenon or an artifact engendered by better methods for recognizing unusual genotypes, *Pectobacterium* spp. collected by one of us (S. H. De Boer) as *E. carotovora* strains from various researchers during 1970 to 1985 were recharacterized by PCR and MLST. Interestingly, a few strains from both New and Old World sources were identified as *P. carotovorum* subsp. *brasiliensis* and a few strains from the United States as *P. wasabiae* (Table 4).

Virulence testing of *Pectobacterium* isolates. The selection of *Pectobacterium* isolates obtained in this study and tested for maceration ability included two strains of *P. atrosepticum*, three of *P. wasabiae*, four of *P. carotovorum* subsp. *brasiliensis*, and two of *P. carotovorum* subsp. *carotovorum.* All isolates tested caused soft rot lesions at the point of inoculation of green pepper fruit and potato tubers. Although the amount of maceration incited by the *P. atrosepticum* and *P. wasabiae* strains (mean diameter of decay in potato = 17 mm) was, on average, less than decay incited by *P. carotovorum* subsp. *brasiliensis* and *P. carotovorum* subsp. *carotovorum* strains (mean diameter of decay = 20 and 21 mm, respectively), the difference was not statistically significant due, in part, to the variability among strains within taxa. In contrast, virulence of *P. atrosepticum* and *P. wasabiae* strains tended to exceed that of *P. carotovorum* subsp. *brasiliensis* and *P. carotovorum* subsp. *carotovorum* when inoculated into potato stems (Fig. 2). The characteristics of the decay lesions on stems and pattern of leaf wilting, except for their severity, were similar among those induced by the different *Pectobacterium* spp. and subspecies.

Analysis of *Pectobacterium* spp. infecting potato plants and tubers grown from inoculated seed tubers in field trials. The incidence of blackleg and stem rot disease that developed in

TABLE 2. Number of different field sources from which stem samples with blackleg symptoms were obtained and the *Pectobacterium* taxa detected therein, and number of individual stems tested from those sources and *Pectobacterium* taxa detected in the individual stems during 2007–2009[a]

	Field sources					Individual stems				
Year	Total	Pa	Pw	Pcbr	Pcc	Total	Pa	Pw	Pcbr	Pcc
2007	11	10	1	1	2	17	11	5	1	1
2008	7	7	2	0	4	30	30	2	0	6
2009	12	10	8	1	1	43	36	25	3	4
Total	30	27 (90%)	11 (37%)	2 (7%)	7 (23%)	90	77 (86%)	32 (36%)	4 (4%)	11 (12%)

[a] Pa = *Pectobacterium atrosepticum*, Pw = *P. wasabiae*, Pcbr = *P. carotovorum* subsp. *brasiliensis*, and Pcc = *P. carotovorum* subsp. *carotovorum*.

TABLE 3. Characteristics of *Pectobacterium* isolates from blackleg-infected potato stems and associated tubers with decay lesions

	Presumptive or known ID[a]												
	Stem isolates					Tuber isolates			Reference strains				
Characteristics	Pa	Pcbr	Pcc	Pw	D	Pa	Pcbr	Pcc	Pa	Pcbr	Pcc	Pw	D
Number of isolates or *strain name*	17	3	3	5	2	2	1	3	*Str31*	*Str212*	*Str71*	*StrS94*	*StrD4*
Growth at 36°C	–	+	+	–	+	–	+	+	–	+	+	–	+
Acid from α-methylglucoside	+	–	–	–	–	+	–	–	+	+	–	–	–
Reducing substances from sucrose	+	–	–	–	–	+	–	–	+	+	–	–	–
Phosphatase activity	–	–	–	–	+	–	–	–	–	–	–	–	+
Taxon PCR[b]	Pa	Pcbr	Pcc	Pw	D	Pa	Pcbr	Pcc	Pa	Pcbr	Pcc	Pw	D
MLST clade[c]	V	I	II	III	ND	V	I	II	V	I	II	III	ND

[a] Pa = *Pectobacterium atrosepticum*, Pcbr = *P. carotovorum* subsp. *brasiliensis*, Pcc = *P. carotovorum* subsp. *carotovorum*, Pw = *P. wasabiae*, and D = *Dickeya* sp.
[b] Positive polymerase chain reaction (PCR) amplification with indicated taxon-specific primer pair.
[c] MLST = multi-locus sequence typing. Cluster identity in phylogenic dendrogram based on housekeeping gene sequences sensu Ma et al. (31); ND designates not done.

plants grown from inoculated seed potato was greater in 2011 than in 2010, probably because of the high rainfall during the 2011 growing season (Table 5). In both years, the incidence of disease was greater in the irrigated than the unirrigated plots. Because disease incidence in many of the treatments was low, data from all treatments inoculated with the same species or subspecies were combined (Table 5). Overall incidence of blackleg-like symptoms was greatest in *P. atrosepticum* treatments, followed by *P. wasabiae*. Plots with *P. carotovorum* subsp. *brasiliensis* and *P. carotovorum* subsp. *carotovorum* treatments had low incidence of disease in both the 2010 and 2011 field trials. Disease symptoms were not distinguishably different among plants in the different treatments.

Multiple *Pectobacterium* spp. were detected by taxon-specific PCR in most diseased stems collected from the 2010 field plot. The presence of noncorresponding *Pectobacterium* spp. (i.e.,

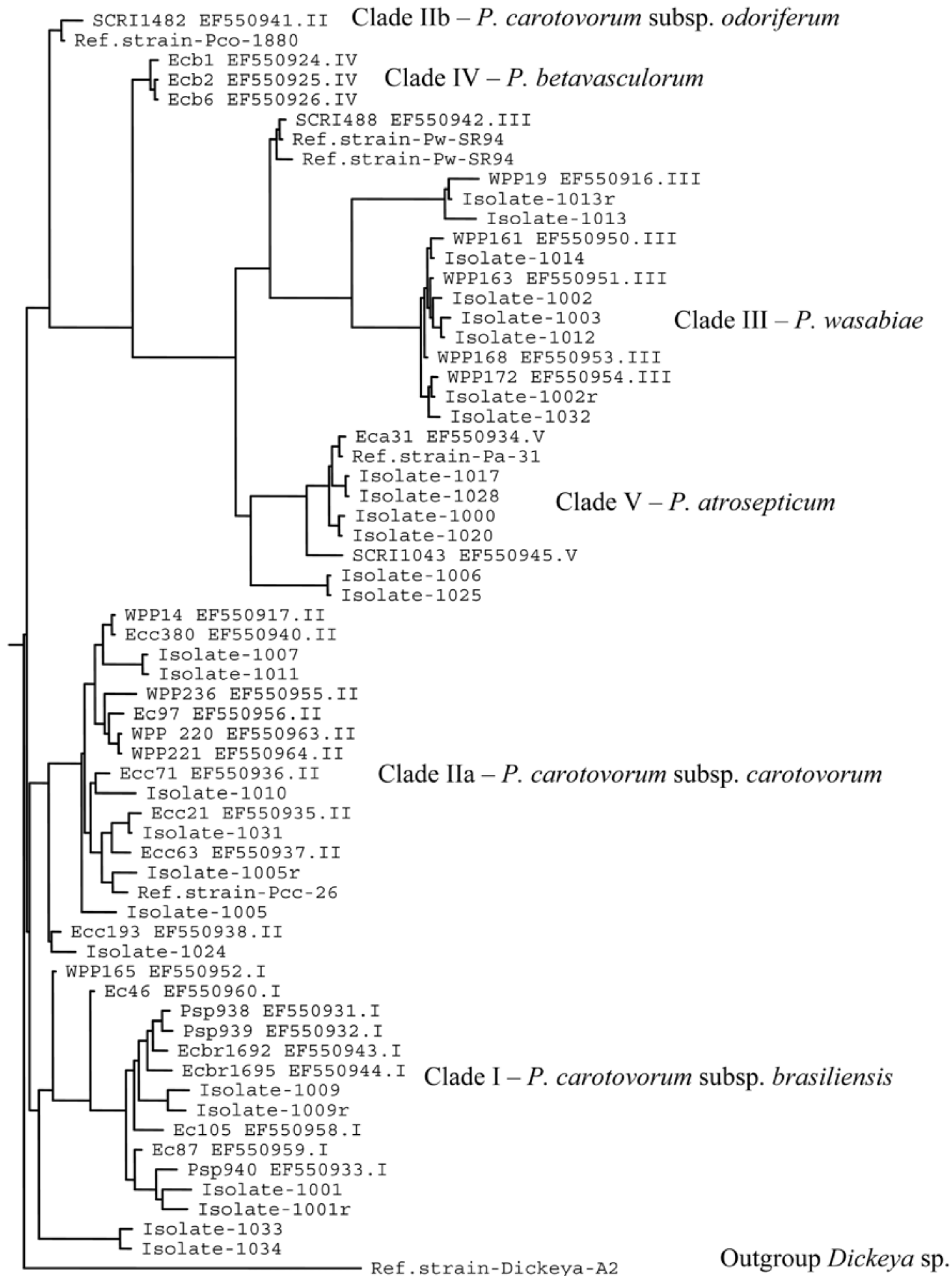

Fig. 1. Neighbor-joining cladogram based on *proA* gene sequences. Sequences obtained from GenBank identified by strain and accession numbers followed by clade designation sensu Ma et al. 2007 (31). Isolates designated with "r" indicate data based on replicate sequencing experiment to validate robustness of clustering of strains based on unedited sequence data.

Pectobacterium spp. not inoculated in the treatment) in diseased stems from all treatments probably resulted from aerial spread of bacteria from diseased plants in adjacent plots; however, the possibility of other sources of inoculum such as the seed tubers cannot be excluded, although no *Pectobacterium* spp. could be detected by PCR in seed tuber sample extracts. In any case, for this reason, treatments were not randomized in the 2011 field trial but, rather, each treatment was surrounded by unplanted spaces and separated by a control treatment. All diseased stems tested from *P. atrosepticum*-inoculated tubers in both the irrigated and unirrigated 2011 trial were positive in the PCR assay for *P. atrosepticum* and negative in the assay for all the other *Pectobacterium* spp., except for one stem that was also positive for *P. carotovorum* subsp. *carotovorum* (Table 6). Of the 25 tested diseased stems grown from *P. wasabiae*-inoculated tubers, 22 were positive in the PCR for *P. wasabiae* and negative in the PCR assays for *P. atrosepticum* and *P. carotovorum* subsp. *brasiliensis* (Table 6). The positive reaction of the 15 stems in the *P. carotovorum* subsp. *carotovorum* PCR assay can be attributed to the cross-amplification of *P. wasabiae* in this assay. The negative PCR reaction of the five diseased stems from the *P. wasabiae* treatment is likely caused by PCR inhibitors in the DNA extracts. Few plants grown from *P. carotovorum* subsp. *brasiliensis*-inoculated seed developed disease, and the subspecies was detected in only three of the eight diseased stems tested (Table 6). However, these stems were also negative in the PCR for the other *Pectobacterium* spp. One of the four diseased stems tested from the *P. carotovorum* subsp. *carotovorum* treatment was positive in the *P. atrosepticum*-specific PCR assay but the likely causal agent of the disease in the other stems, being negative in the other PCR assays, was not ascertained (Table 6).

P. wasabiae was successfully isolated from diseased stems of plants grown from seed tubers inoculated with each of the two isolates. However, *P. carotovorum* subsp. *brasiliensis* could not be isolated from corresponding diseased stems even though its presence was detected in the taxon-specific PCR assay, perhaps because the stems were stored for several months at –20°C prior to attempting isolation. No attempt was made to isolate *Pectobacterium* spp. from diseased stems of the *P. atrosepticum* and *P. carotovorum* subsp. *carotovorum* treatments.

P. atrosepticum and *P. wasabiae* were detected by taxon-specific PCR in at least one composite sample of progeny tubers harvested from corresponding plots in the 2010 unirrigated field trial. Similarly, *P. carotovorum* subsp. *brasiliensis* and *P. wasabiae* but not *P. atrosepticum* was detected in at least one composite tuber sample from corresponding plots in the 2011 unirrigated field trial. *P. atrosepticum*, *P. carotovorum* subsp. *brasiliensis* and *P. wasabiae* were all detected in corresponding

TABLE 4. Reidentification of *Pectobacterium carotovorum* strains in a laboratory culture collection of *Pectobacterium* spp. isolated during 1970–1985

		Number of strains identified per taxon[a]		
Geographic origin of strains	Number of strains	*P. carotovorum* subsp. *carotovorum*	*P. carotovorum* subsp. *brasiliensis*	*P. wasabiae*
British Columbia, Canada	46	44	2	0
Oregon, United States	26	23	2	1
Wisconsin, United States	49	44	3	2
Other states, United States	7	6	0	1
Scotland	31	28	3	0
The Netherlands	36	35	1	0
Peru	5	3	2	0
Total	200	183	13	4

[a] Strains identified on basis of taxon-specific PCR and confirmed by multi-locus sequence typing for strains identified as *P. carotovorum* subsp. *brasiliensis* and *P. wasabiae*.

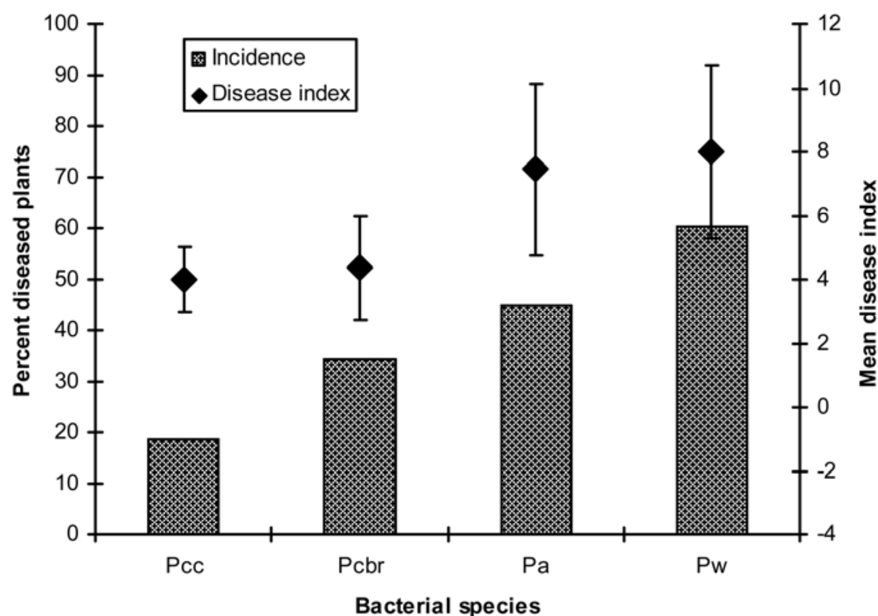

Fig. 2. Disease development in stem-inoculated, greenhouse-grown potato plants indicated by incidence as percentage of plants developing symptoms (disease index >2) and disease severity as the mean of disease index of diseased plants only at 11 days after inoculation. Data points based on inoculations of two plants of each of four potato cultivars ('Fabula', 'Norland', 'Superior', and 'Yukon Gold') for two strains of *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc) (n = 16), four strains of *P. carotovorum* subsp. *brasiliensis* (Pcbr) (n = 32), five strains of *P. atrosepticum* (Pa) (n = 40), and six strains of *P. wasabiae* (Pw) (n = 48). Disease index (0 to 10) is the sum of scores for decay and for wilting each based on a scale from 0 (no decay or wilt) to 5 (extensive decay or wilt).

composite tuber samples from irrigated plots of both 2010 and 2011. *P. carotovorum* subsp. *brasiliensis* was detected in at least one composite sample of noncorresponding composite tuber samples from irrigated plots of both 2010 and 2011, except from the *P. atrosepticum*-inoculated treatment in 2010. *P. carotovorum* subsp. *brasiliensis* was also detected most frequently in PCR assays of individual tuber samples from the 2010 irrigated plot, although *P. atrosepticum* and *P. wasabiae* were also detected in tubers from noncorresponding treatments (Table 7). To be sure that the apparent widespread presence of *P. carotovorum* subsp. *brasiliensis* in progeny tubers was not a PCR artifact, the PCR result was confirmed for two samples by sequencing the PCR amplicon and for three samples by isolation of the bacterium. PCR tests for *P. carotovorum* subsp. *carotovorum* were not done on progeny tuber samples.

DISCUSSION

The number of species and subspecies of *Pectobacterium* has increased over recent years and, as a result, their identification and differentiation by classical microbiological tests has become increasingly challenging. Previously, *P. atrosepticum* and *P. carotovorum* were the most frequently encountered species in soft rot disease of plants and were readily distinguished by whether they grew at 36°C and whether or not they could produce acid from α-methylglucoside and reducing substances from sucrose. With the recognition of additional species and subspecies, including *P. betavasculorum*, *P. wasabiae*, *P. carotovorum* subsp. *brasiliensis*, and *P. carotovorum* subsp. *odoriferum*, it has become more difficult to make accurate identifications based on biochemical tests alone because carbohydrate utilization patterns and other phenotypic characteristics vary among strains of the same species or subspecies. However, the differentiation of *Pectobacterium* spp. by MLST into clades which closely correspond to described species and subspecies (31) provides a convenient means for determining or confirming the affiliation of new isolates. The rapidity and low cost of DNA sequencing makes MLST a good alternative to classical determination of physiological and biochemical characteristics for bacterial identification. Our results indicated that complete MLST analysis on multiple concatenated gene sequences was unnecessary to identify isolates, because sequence analysis of even a single housekeeping gene provided good identification when evaluated in combination with an adequate number of selected GenBank sequences of the same gene to define the *Pectobacterium* spp. and subspecies. However, to insure correct identification, we preferred to sequence at least two housekeeping genes to identify new isolates. The strategy of using housekeeping gene sequences for identifying new isolates of *Pectobacterium* spp. and the closely related *Dickeya* spp. from potato has also already been used by other researchers (1, 37, 39).

Molecular techniques have not only contributed to the facile and rapid identification of bacterial isolates but their use as

TABLE 5. Incidence of blackleg-like symptoms in potato plants ('Superior') grown in field plots from seed tubers inoculated by vacuum infiltration with cultures of *Pectobacterium atrosepticum*, *P. carotovorum* subsp. *carotovorum, P. carotovorum* subsp. *brasiliensis, P. wasabiae*, or sterile distilled water

		Plants with blackleg symptoms (%) per seed tuber inoculant				
Year	Irrigation	*P. atrosepticum*	*P. carotovorum* subsp. *brasiliensis*	*P. carotovorum* subsp. *carotovorum*	*P. wasabiae*	Water
2010	No	4	3	0	1	0
2010	Yes	13	5	2	8	0
2011	No	36	5	3	11	0
2011	Yes	47	1	5	13	0

TABLE 6. *Pectobacterium* spp. and subspecies detected by taxon-specific polymerase chain reaction (PCR) in diseased stems of potato plants grown in a field trial from inoculated seed tubers during 2011

		Number of stems positive in PCR[a]			
Inoculated taxon	Number of stems tested	*Pectobacterium atrosepticum*	*P. carotovorum* subsp. *brasiliensis*	*P. carotovorum* subsp. *carotovorum*[b]	*P. wasabiae*
Unirrigated trial					
P. atrosepticum	9	9	0	1	0
P. carotovorum subsp. *brasiliensis*	4	0	1	0	0
P. carotovorum subsp. *carotovorum*	2	1	0	0	0
P. wasabiae	13	0	0	8	11
Irrigated trial					
P. atrosepticum	8	8	0	0	0
P. carotovorum subsp. *brasiliensis*	4	0	2	0	0
P. carotovorum subsp. *carotovorum*	2	0	0	0	0
P. wasabiae	12	0	0	7	11[c]

[a] *Pectobacterium* spp. detected by PCR.
[b] PCR assay for *P. carotovorum* subsp. *carotovorum* cross-reacted with *P. wasabiae*.
[c] *P. wasabiae* was isolated and characterized from three of these stems.

TABLE 7. Incidence of *Pectobacterium* spp. and subspecies detected by taxon-specific polymerase chain reaction (PCR) in stolon end tissue of individual progeny tubers harvested from the 2010 irrigated field trial

		Tubers positive in PCR (%)[a]		
Inoculated taxon	Number of tubers tested	*Pectobacterium atrosepticum*	*P. carotovorum* subsp. *brasiliensis*	*P. wasabiae*
P. atrosepticum	30	13	40	10
P. carotovorum subsp. *brasiliensis*	20	0	30	5
P. carotovorum subsp. *carotovorum*	20	20	25	0
P. wasabiae	30	7	23	30
Control (water)	10	0	30	10

[a] *Pectobacterium* spp. detected by PCR.

detection tools also provides an opportunity to assess the presence of multiple bacteria in complex environments without the need for isolation. The availability of taxon-specific PCR assays for the *Pectobacterium* spp. allowed evaluation of the mixture of *Pectobacterium* spp. present in decayed potato tissue. The specificity of the PCR assays using the *P. atrosepticum* and *P. carotovorum* subsp. *brasiliensis* primers were confirmed in preliminary PCR tests on characterized pure cultures. Specificity of PCR with the *P. carotovorum* subsp. *carotovorum* primer pair was more limited because they also amplified the expected 550-bp product from some strains identified as *P. wasabiae* (25) and did not amplify DNA from other strains that were identified as *P. carotovourm* subsp. *carotovorum* on the basis of biochemical tests. The isolation of *P. wasabiae* strains early on in this study necessitated the development of a specific primer set for this species and was accomplished using GenBank sequences of the phytase gene (44). Subsequently, a new *P. wasabiae*-specific PCR assay using newly designed primers based on the YD repeat protein gene was published (27). Specificity of these primers (PW7011F/R) was identical to those of the phytase-directed primers designed in this study when compared in tests with pure culture and sample DNA.

The presence of multiple *Pectobacterium* spp. in potato, even in the same plant, has been noted previously (26,31). In our study, we specifically targeted potato stems with typical blackleg symptoms and, although most stems were positive for *P. atrosepticum* in PCR, as expected if this species continues to be the main cause of potato blackleg in Canada, in only one-half of the stems was it the only *Pectobacterium* sp. detected. Interestingly, *P. wasabiae* was detected as the only *Pectobacterium* sp. present in several stems but was also present most frequently in combination with *P. atrosepticum*. Additionally, *P. carotovorum* subsp. *carotovorum* and, occasionally, *P. carotovorum* subsp. *brasiliensis* were detected in stems along with *P. atrosepticum* or *P. wasabiae*. Although the *Pectobacterium* spp. or subspecies isolated from symptomatic blackleg stems, with one exception, were among those that had been detected by PCR in the same samples, the molecular approach to evaluating the microbial populations revealed a more complex soft-rotting microbial population than is usually evident from evaluations based on isolation alone. The presence of multiple *Pectobacterium* spp. in diseased plants raises the specter of synergism among genotypes that might exacerbate incidence or severity of disease. The question of synergistic activity among the pectolytic bacteria is yet to be fully addressed but, in a preliminary experiment in which both *P. atrosepticum* and *P. wasabiae* were vacuum inoculated into seed tubers, disease incidence in the subsequent plant stand was not greater than when either pathogen was inoculated alone (*unpublished observation*).

P. wasabiae was previously implicated as a cause of potato tuber rots in New Zealand, Iran, and Wisconsin and was shown to have capacity for causing decay lesions in stems upon artificial inoculation (1,31,39); however, this study is the first to show that it is also a causal agent of a blackleg-like disease in potato. *P. wasabiae* was isolated as the sole *Pectobacterium* sp. from a number of potato stems expressing typical blackleg symptoms collected from commercial potato fields. Inoculation of greenhouse-grown plants confirmed its capacity to cause stem decay and, in field trials, *P. wasabiae*-inoculated tubers developed plants with typical blackleg symptoms. Detection in and reisolation from diseased stems fulfilled Koch's postulates for confirming disease etiology. The vacuum infiltration method of inoculating seed potato tubers with *Pectobacterium* spp. simulated natural tuber contamination and served to show that *P. wasabiae* induced stem disease from tuber-borne inoculum under agricultural field conditions apart from causing stem decay lesions from artificial inoculation of aboveground tissue. A similar tuber vacuum infiltration strategy was used to show that some strains of *P. carotovorum* subsp. *carotovorum* also causes blackleg in potato under temperate climatic conditions (14).

The Canadian isolates of *P. carotovorum* subsp. *brasiliensis* were clearly less virulent as a potato stem pathogen than isolates of *P. atrosepticum* and *P. wasabiae*. Although a few plants grown from *P. carotovorum* subsp. *brasiliensis*-inoculated seed tubers developed blackleg-like symptoms, *P. carotovorum* subsp. *brasiliensis* could not be isolated from these stems. The low level of virulence exhibited by our *P. carotovorum* subsp. *brasiliensis* isolates contrasts with the high virulence of the Brazilian isolates (15). Although our isolates of *P. carotovorum* subsp. *brasiliensis*, along with those obtained in Wisconsin (31), formed a novel clade in MLST dendrograms with strains from Brazil, they did differ from the Brazilian strains in that they did not produce acid from α-methylglucoside or reducing substances from sucrose. It is evident that virulence characteristics may be quite unrelated to taxonomic placement, and unsurprisingly so, as pointed out by others (55), because modern bacterial taxonomy increasingly relies on the nucleotide sequence of various genomic loci such as 16S rRNA genes and housekeeping genes, chromosomal regions quite separate from pathogenicity and virulence genes. Moreover, tuber maceration ability, although a characteristic of all the *Pectobacterium* strains, also varied in aggressiveness among strains of the same species or subspecies and was not correlated with blackleg virulence. These results are consistent with those of Kim et al. (26), who also found that there was no correlation between maceration ability and possession of a purported pathogenicity factor, the type III secretion system.

The migration of *P. atrosepticum* bacteria into progeny tubers via the stolons has been a hallmark of the blackleg disease. In this article, we provide experimental evidence that *P. wasabiae* and *P. carotovorum* subsp. *brasiliensis* also occur in the stolon attachment site of progeny tubers developing on plants grown from inoculated seed tubers. Infection of progeny tubers via stolons is an important means by which *P. atrosepticum* is known to be propagated from one generation of potato production to the next, whereas field inoculum is considered unimportant because the species survives poorly in the soil environment and is not known to be associated with weed hosts or to persist in other environmental niches. This may be different for *P. carotovorum* subsp. *brasiliensis* and *P. wasabiae*. The capacity of these species to persist in the field environment has not been studied to any extent but, if their environmental survival capacity is greater than that of *P. atrosepticum* such as *P. carotovorum* subsp. *carotovorum*, they potentially could also colonize potato plants directly from soil inoculum reservoirs, as was elegantly demonstrated for *Dickeya* spp. recently (5).

The persistence and behavior of bacteria in the field environment has consequences for disease control strategies. Whether or not plant-pathogenic bacteria persist in soil between growing seasons, survive in association with weed plants, are present in irrigation water, or are readily dispersed in atmospheric aerosols will have consequences for the success of disease control strategies that utilize pathogen-tested minitubers or aseptic tissue cultures to initiate each cycle of seed potato propagation. The rate of contamination with *Pectobacterium* spp. once the potato tubers are planted in the field is, in part, a function of the capacity of the bacteria to persist in the environment, as well as its propensity to spread through contamination of agricultural equipment (55). Moreover, the capacity of pectolytic bacteria to survive on stored seed potato tubers and their optimum temperature for initiation of soft rot lesions will affect the storability of crops and management practices for minimizing storage loss. The deliberate strategies for controlling blackleg caused by *P. atrosepticum*, supported by a large number of studies published in the scientific literature and promulgated in extension publications, may not be directly applicable for controlling diseases caused by *P. carotovorum* subsp. *brasiliensis* or *P. wasabiae*. To avoid confusion and mis-

application of information pertaining specifically to *P. atrosepticum*-caused blackleg, it is suggested that use of the blackleg name for the diseases caused by *P. carotovorum* subsp. *brasiliensis*, *P. carotovorum* subsp. *carotovorum*, and *P. wasabiae* be avoided despite the similar symptomologies. Rather, all bacterial rots of potato stems could more appropriately be designated under a single name such as "bacterial stem rot". The bacterial stem rot nomenclature can equally well accommodate potato stem rots caused by the various *Dickeya* spp. as it can accommodate stem rots caused by *Pectobacterium* spp. because all have overlapping symptomologies (55). A previous recommendation (38) that all potato stem rots be named "blackleg" to facilitate visual disease rating by crop inspectors as a requirement for seed potato certification programs blurs the fact that much of what has been written in the past about blackleg refers specifically to disease caused by *P. atrosepticum.* Coining a new functional term for all bacterial stem rots of potato, including blackleg and diseases with similar symptoms, facilitates the terminology for inspectors and producers.

This study confirmed that *P. atrosepticum* remains the most important causal agent of blackleg disease in Canada, similar to results in Finland, where *P. atrosepticum* remains the major cause of blackleg despite the increasingly common presence of *Dickeya* spp. in potato stocks (28). In other parts of Western Europe, New Zealand, Brazil, and South Africa, *P. atrosepticum* is no longer or perhaps never was the primary cause of potato blackleg. The shift in potato stem rot etiology from a disease caused by essentially a single serotype of the one species, *P. atrosepticum*, to a disease syndrome involving several distinct taxa of soft-rotting bacteria, as observed in this study, is quite remarkable but not as dramatic as the shift in Western Europe, where potato stem rot is now almost entirely due to *Dickeya* spp. (6,55). In both North America and Western Europe, this etiological shift follows an extended period of approximately 30 years during which significant changes in the potato industry also took place. In the past, seed potato tubers were grown from clonally selected field tubers on which a stable microbial community was maintained and passed on from one generation of potato production to the next. Populations of specific genotypes of *P. atrosepticum* and *P. carotovorum* subsp. *carotovorum*, well adapted to the potato biosphere, were most frequently associated with the potato crop. Serogroup I was, by far, the most predominate strain of *P. atrosepticum*, and serogroup III of *P. carotovorum* subsp. *carotovorum* predominated (8,10). An earlier study also found that populations of *Pectobacterium* spp. on potato foliage that presumably were deposited from environmental sources had little impact on the make up of belowground populations in the plant root zone (8), supporting the long-held view that the best-adapted strains persist in association with host crop plants (49). Whether field temperature during the growing season or other factors determine which strains in the *Pectobacterium* sp. complex are best adapted to a particular crop is not known.

Today, seed potato crops are mainly initiated from minitubers propagated in protected environments from tissue culture plantlets and then multiplied for a limited number of field generations. Minitubers that are planted to produce the first field generation of seed potato are microbiologically naïve and susceptible to colonization by bacteria from the environment. The best-adapted and most competitive bacterial species have the potential of colonizing progeny tubers and remain associated with the seed crop during subsequent generations of potato production. In Canada and the United States, strains of the new-found *Pectobacterium* taxa, *P. wasabiae* and *P. carotovorum* subsp. *brasiliensis*, perhaps are sometimes among the primary colonizers of first-field-generation seed potato. In Western Europe, *Dickeya* spp. is an important primary colonizer of microbiologically naïve early-generation potato seed stocks (55). More recently, the so-called *D. solani* strain, which is very closely related to a strain occurring on hyacinth in the Netherlands, contaminates European seed potato crops (7,55), and it is speculated that intercrop movement of specific bacterial strains occurs readily via irrigation water (7). This would explain the specific association of *Dickeya* spp. with seed potatoes imported from the Netherlands, where a vigorous seed potato industry thrives in close proximity to an extensive flower bulb industry (57,58). In light of these observations, artificial inoculation of microbiologically naïve minitubers with a low virulence strain of *P. carotovorum* subsp. *carotovorum* to occupy the *Pectobacterium* niche of the potato biosphere may be an interesting disease control strategy to avoid colonization with more virulent or aggressive strains of other soft rot bacteria.

The fact that some strains of *Pectobacterium* spp. isolated more than 30 years ago from potato in both North America and Western Europe can now be identified as *P. carotovorum* subsp. *brasiliensis* and *P. wasabiae* (Table 4) proves that these strains are not new to potato. Although their identification has only been made possible by recent developments in molecular methods, it is also notable that their number is small in comparison with the total number of isolates evaluated. Discovery of the role that these genotypes play in potato stem rots is probably a result not only of the availability of the new tools for their identification but also of their increased frequency due to the changes in potato production practices, as described above. Whether the assortment of *Pectobacterium* spp. and subspecies associated with the potato crop affects the incidence of disease in the field, the susceptibility of the crop under different growing conditions due to the differences in growth condition optima, or susceptibility to decay in storage remains to be determined. Certainly, greater attention needs to be paid to strain identity and the combination of strains when studying bacterial stem rots of potato than had been previously recognized.

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