Journal of Applied Microbiology 2004, **96,** 535–545 doi:10.1111/j.1365-2672.2004.02173.x

Characterization of atypical *Erwinia carotovora* strains causing blackleg of potato in Brazil

V. Duarte[1], S.H. De Boer[2], L.J. Ward[2] and A.M.R. de Oliveira[1]

[1]Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil, and [2]Canadian Food Inspection Agency, Centre for Animal and Plant Health, Charlottetown, PEI, Canada

2003/0497: received 10 June 2003, revised 31 October 2003 and accepted 2 November 2003

ABSTRACT

V. DUARTE, S.H. DE BOER, L.J. WARD AND A.M.R. DE OLIVEIRA. 2004.

Aims: To determine the characteristics of bacteria associated with the blackleg disease of potato in Brazil and compare them with species and subspecies of pectolytic *Erwinia*.

Methods and Results: Biochemical and physiological characteristics of 16 strains from blackleg-infected potatoes in State of Rio Grande do Sul, Brazil, were determined and differentiated them from all the *E. carotovora* subspecies and *E. chrysanthemi*. Pathogenicity and maceration ability of the Brazilian strains were greater than those of *E. carotovora* subsp. *atroseptica*, the causal agent of potato blackleg in temperate zones. Analyses of serological reaction and fatty acid composition confirmed that the Brazilian strains differed from *E. carotovora* subsp. *atroseptica*, but the sequence of 16S rDNA gene and the 16S-23S intergenic spacer (IGS) region confirmed the Brazilian strains as pectolytic *Erwinia*. Restriction analysis of the IGS region differentiated the Brazilian strains from the subspecies of *E. carotovora* and from *E. chrysanthemi*. A unique *Sex*AI restriction site in the IGS region was used as the basis for a primer to specifically amplify DNA from the Brazilian potato blackleg bacterium in PCR.

Conclusions: The bacterium that causes the blackleg disease of potato in Brazil differs from *E. carotovora* subsp. *atroseptica*, the blackleg pathogen in temperate zones. It also differs from other subspecies of *E. carotovora* and from *E. chrysanthemi* and warrants status as a new subspecies, which would be appropriately named *E. carotovora* subsp. *brasiliensis*.

Significance and Impact of the Study: The blackleg disease of potato is caused by a different strain of pectolytic *Erwinia* in Brazil than in temperate potato-growing regions. The Brazilian strain is more virulent than *E. carotovora* subsp. *atroseptica*, the usual causal agent of potato blackleg.

Keywords: blackleg, *Erwinia*, *Pectobacterium*, potato, soft rot, *Solanum tuberosum*.

INTRODUCTION

Erwinia carotovora and *E. chrysanthemi* are the most important of the pectolytic bacteria that cause maceration of plant tissue and disease of many crop plants including potato. Several years ago Hauben *et al.* (1998) revived the suggestion that the pectolytic bacteria be placed in a separate genus, *Pectobacterium*, on the basis of 16S rDNA sequences, but the generic epithet, *Erwinia*, remains the preferred designation in the scientific literature. The *E. carotovora* species is divided into the five subspecies: *atroseptica*, *carotovora*, *betavasculorum*, *odorifera* and *wasabiae* (De Boer and Kelman 2000) some of which should perhaps be elevated to species status (Gardan *et al.* 2003).

The blackleg disease of potato is caused primarily by *E. c. atroseptica* in cool temperate climates. *E. c. carotovora* and *E. chrysanthemi* may also cause blackleg-like symptoms at high temperatures (<25°C) but economically, *E. c. atroseptica* is the most important pathogen (Pérombelon and Kelman 1980; Pérombelon 1992). *Erwinia c. atroseptica* potato isolates from various geographical areas in Canada,

Correspondence to: Valmir Duarte, Universidade Federal do Rio Grande do Sul, Departamento de Fitossanidade, Cx. Postal 15100 – CEP 90001-970, Porto Alegre, RS, Brazil (e-mail: valmir@vortex.ufrgs.br).

the US and western Europe are remarkably uniform in both phenotypic and genetic characteristics (De Boer *et al.* 1987; Fessehaie *et al.* 2002). *Erwinia c. betavasculorum* and *E. c. wasabiae* also define groups of similar strains and cause soft rot of sugar beet and Japanese horseradish, respectively (Thomson *et al.* 1981; Goto and Matsumoto 1987). Some strains, not isolated from potato, have been described as atypical *E. c. atroseptica* because, although they produce acid from α-methylglucoside and reducing substances from sucrose like *E. c. atrospetica*, they differ in having the ability to grow at 37°C. Some of these strains, isolated from several hosts, including chicory, produce odorous volatile metabolites and are called *E. c. odorifera* (Gallois *et al.* 1992).

In a recent study, *E. c. atroseptica*, *E. c. carotovora* and *E. chrysanthemi* were found in 55, 44 and 1%, respectively, of potato plants showing blackleg symptoms in 22 fields of nine counties in Rio Grande do Sul State, Brazil (de Oliveira 2001). The strains associated with blackleg, tentatively identified as *E. c. atroseptica*, had typical colonies that produced reducing sugars from sucrose, utilized α-methylglucoside, and had no phosphatase activity. However, these bacteria grew at 37°C, produced no PCR product with *E. c. atroseptica*-specific primers and did not react with a monoclonal antibody specific to serogroup I of *E. c. atroseptica*. These results, suggesting that the pectolytic bacteria causing blackleg in potato differed from *E. c. atroseptica*, prompted us to investigate further the characteristics of these strains and their differences from described subspecies of *E. carotovora*.

MATERIALS AND METHODS

Strains and biochemical characterization

The strains investigated in this study are listed in Table 1. Sixteen strains of the Brazilian potato blackleg-causing bacterium (BPBB) were chosen from among the 400 isolates with *E. c. atroseptica*-like characteristics to represent three potato cultivars and two major production areas in the state of Rio Grande de Sol. The bacteria had been isolated in 1999 from potato plants showing blackleg symptoms (de Oliveira 2001). Four of the selected strains (8, 212, 213 and 371) were deposited in the American Type Culture Collection (BAA-416, 417, 418 and 419, respectively). Strains were routinely maintained on nutrient agar (NA).

Strains were characterized by standard tests used for pectolytic *Erwinia* and included a test for pectolytic activity on crystal violet pectate (CVP) medium, acid production from α-methylglucoside, reducing substances from sucrose, growth at 37°C, and erythromycin resistance (De Boer and Kelman 2000). The MICRO-ID kit (Remel Inc., Lenexa, KS, USA) that tests 15 additional metabolic activities was used for further characterization.

Strains were also assayed for oxidation of the 95 carbon sources on the GN2 Biolog microplates and an additional 45 carbon sources on the GP microplates (Biolog Inc., Hayward, CA, USA). Bacteria were grown on general medium (BUGM, Biolog Inc.) for 24 h at 24°C. Using sterile cotton swabs, bacteria were transferred from the medium surface to 30-ml test tubes containing 20 ml of inoculating fluid with thioglycolate (Biolog Inc.). The optical density of the suspension was adjusted as recommended by the manufacturer. Microplates were inoculated with 150 μl of suspension per well and incubated at 24°C. Absorbancy was determined at 590 nm after 24 h using Biolog Microlog 2 Workstation and the GN database (release 4·01C) for strain identification.

Biochemical data were evaluated by cluster analysis using the single linkage method (nearest neighbour) using SYSTAT software (Chicago, IL, USA). The data from 67 and 26 carbon sources on GN and GP Biolog plates, respectively, utilized by at least one strain, were included in the analysis. Data were scored zero for negative reaction, 0·5 for borderline reactions, and 1 for positive results.

Pathogenicity and maceration characteristics

To obtain host plants for testing pathogenicity, seed potatoes were planted in 12-cm-diameter pots containing a potting soil substrate (Growing mix #5; Northern Peat Ltd, Berwick, NS, CA, USA). One seed tuber was planted per pot and four pots were used for each bacterial strain tested. Plants were kept in a growth chamber at 21°C, and stems were inoculated 20 days after planting. Inoculation was carried out with sterile toothpicks dipped into 24–48-h-old bacterial colonies and then immediately stabbed into individual potato stems, 5 cm above the soil line, at three stems per pot. Plants were observed for blackleg symptoms for 21 days after inoculation.

To test maceration ability, sterile toothpicks dipped into 24–48-h-old bacterial colonies were stabbed into potato tubers. After inoculation, tubers were sprayed with mineral oil (Nujol) (Lee and Cha 2001) and kept in a humid chamber at 24°C. After incubation for 5 days, the amount of macerated tissue was determined by weighing tubers before and after washing away decayed tissue.

Green peppers, previously disinfected with 70% alcohol and 1% NaOCl for 30 s each, and rinsed with sterile distilled water, were inoculated in the same way as potato tubers, but incubated without oil spray. After 48 h at 24°C in a humid chamber, the diameter of decay lesions were measured at each inoculation locus.

Table 1 Strains of the Brazilian potato blackleg bacterium (BPBB) and *Erwinia* species and subspecies used in this study

Species/subspecies	Identification	Potato cultivar, region in RS, Brazil	Reference/source
BPBB	8 (ATCC BAA-416)	Elvira, Planalto	de Oliveira (2001)/UFRGS 32·338
	29	Elvira, Planalto	de Oliveira (2001)/UFRGS32 ·333
	54	Baronesa, Planalto	de Oliveira (2001)/UFRGS 32·025
	101	Macaca, Depressao Central	de Oliveira (2001)/UFRGS 32·041
	106	Macaca, Depressao Central	de Oliveira (2001)/UFRGS 32·064
	137	Baronesa, Depressao Central	de Oliveira (2001)/UFRGS 32·006
	138	Baronesa, Depressao Central	de Oliveira (2001)/UFRGS 32·038
	142	Baronesa, Planalto	de Oliveira (2001)/UFRGS 32·402
	153	Macaca, Planalto	de Oliveira (2001)/UFRGS 32·358
	200	Baronesa, Depressao Central	de Oliveira (2001)/UFRGS 32·031
	201	Macaca, Depressao Central	de Oliveira (2001)/UFRGS 32·085
	205	Macaca, Depressao Central	de Oliveira (2001)/UFRGS 32·074
	212 (ATCC BAA-417)	Elvira, Planalto	de Oliveira (2001)/UFRGS 32·401
	213 (ATCC BAA-418)	Macaca, Depressao Central	de Oliveira (2001)/UFRGS 32·084
	219	Elvira, Planalto	de Oliveira (2001)/UFRGS 32·413
	371 (ATCC BAA-419)	Macaca, Depressao Central	de Oliveira (2001)/UFRGS 32·062
		Serogroup of potato strain	
E. carotovora subsp. *atroseptica*	Eca 3	I	S.H. De Boer/3
	Eca 6	XVIII	R. J. Copeman/E17
	Eca 17	I	S.H. De Boer/17
	Eca 19	I	S.H. De Boer/19
	Eca 31	I	A. Kelman/SR8
	Eca 196	XXII	S.H. De Boer/196
	Eca 198	XX	R.J. Copeman/E555
		Host	
E. carotovora subsp. *betavasculorum*	LMG 2398	Sugar beet	Thomson *et al.* (1981)
	LMG 2461	Sugar beet	Thomson *et al.* (1981)
	LMG 2462	Sugar beet	Thomson *et al.* (1981)
	LMG 2464^{T}	Sugar beet	Thomson *et al.* (1981)
		Serogroup of potato strain	
E. carotovora subsp. *carotovora*	Ecc 21	XXIX	H.P. Maas Geesteranus/139
	Ecc 23	XV	H.P. Maas Geesteranus/162
	Ecc 26	V	H.P. Maas Geesteranus/200
	Ecc 51	XIV	S.H. De Boer/51
	Ecc 59	XIII	H. P. Maas Geesteranus/257
	Ecc 61	X	S.H. De Boer/222
	Ecc 62	IX	S.H. De Boer/195
	Ecc 63	IX	S.H. De Boer/202
	Ecc 65	XIV	S.H. De Boer/196
	Ecc 67	XII	A. Kelman/SR162
	Ecc 68	VII	A. Kelman/SR165
	Ecc 71	III	H.P. Maas Geesteranus/226
	Ecc 94	XVII	R.J. Copeman/E6
	Ecc 193	XI	R.J. Copeman/E193
	Ecc 194	XIX	R.J. Copeman/E103
		Host	
E. carotovora subsp. *odorifera*	CFBP 1645	Celery	R. Samson (1978)
	CFBP 1878^{T}	Witloof chicory	R. Samson (1978)
	CFBP 1880	Witloof chicory	R. Samson (1979)
	CFBP 1893	Celery	R. Samson (1976)
E. carotovora subsp. *wasabiae*	Ecw SR91^{T}	Horseradish	Goto and Matsumoto (1987)
	Ecw SR92	Horseradish	Goto and Matsumoto (1987)
	Ecw SR93	Horseradish	Goto and Matsumoto (1987)
	Ecw SR94	Horseradish	Goto and Matsumoto (1987)
E chrysanthemi	Ech 571	Potato	H.P. Maas Geesteranus/647

ATCC, American Type Culture Collection; LMG, Laboratorium Microbiologie Rijksuniversiteit Gent; CFBP, Collection Francaise des Bacteries Phytopathogenes.

Serological and fatty acid analyses

The monoclonal antibody 4F6 specific for the lipopolysaccharide of serogroup I, the principle serological type of *E. c. atroseptica*, was tested by ELISA as described previously (De Boer and McNaughton 1987).

Gas chromatography of fatty acid methyl esters (FAME) using the Sherlock Microbial Identification System (MIDI, Inc., Newark , DE, USA) was conducted by R. Phillipe at the Centre for Plant Quarantine Pests, Ottawa, Canada.

DNA sequencing and restriction digestion

The 16S rDNA fragment from selected BPBB strains amplified with the 27f and L1r consensus primers were submitted for sequencing as described (Fessehaie *et al.* 2002). Sequencing was carried out by Dr L. Wong at the Core Molecular Biology Facility, York University (North York, Ontario, Canada).

DNA from the intergenic spacer (IGS) flanking the 3′ end of the 16S and the 5′ end of the 23S rRNA genes was obtained by the 'band stab' amplification technique, in which resolved PCR products, based on the 1491f (5′-GAA GTC GTA ACA AGG TA-3′) and L1r [5′-CA(A/G) GGC ATC CAC CGT-3′] primers, were retrieved directly from an agarose gel with a pipet tip after electrophoresis and re-amplified (Stackebrandt and Goodfellow 1991; Wilton *et al.* 1997). PCR products were purified using micro-CLEAN (The Gel Company, San Francisco, CA, USA) and sequenced as described above. Alignments of the sequences were performed using Align Plus 4, version 4·1 (Sci Ed Central, San Francisco, CA, USA).

As a way of measuring the genetic diversity of BPBB strains and differentiation from other subspecies, fingerprints of the IGS regions were generated from strains that were isolated from different locations in RS State, Brazil, and characterized in detail. PCR amplifications were carried out as previously reported (Fessehaie *et al.* 2002). The amplification was carried out with 1 μM each of primers 1491f and L1r, using a thermal regime of 94°C/2 min, 25x (94°C/45 s, 62°C/45 s, 72°C/90 s), and 72°C/10 min. Purified amplicons were digested with *Rsa*I, *Cfo*I, *Hpy*-CH4III, and *Sex*AI (BioLabs Inc., New England, CT, USA) and DNA fragments were resolved by gel electrophoresis on 3% agarose gels.

PCR amplification

For each strain evaluated, genomic DNA was extracted using a protocol adapted from De Boer and Ward (1995). Briefly, a loopful of bacterial cells was extracted in 250 μl of extraction buffer (100 mM Tris–HCl pH 8·0, 25 mM EDTA, 1% SDS, and 5 μg proteinase K) and incubated for 3 h at 56°C. Protein components were precipitated with one half volume of ammonium acetate (7·5 M) and removed by centrifugation. DNA was precipitated from the supernatant fraction with isopropanol, washed with 70% ethanol, taken up in 50 μl of ultrapure water, and stored at −20°C.

Primers ECA1f (5′-CGG CAT CAT AAA AAC ACG-3′) and ECA2r (5′-GCA CAC TTC ATC CAG CGA-3′) were used in PCR reactions as described previously (De Boer and Ward 1995) but using a TGradient (Whatman-Biometra, Goettingen, Germany) thermocycler. Primers Y1 (5′-TTA CCG GAC GCC GAG CTG TGG CGT-3′) and Y2 (5′-CAG GAA GAT GTC GTT ATC GCG AGT-3′), selected from a pectate lyase-encoding gene of the Y family (Darrasse *et al.* 1994), were also used. PCR amplification was carried out as described but with the following thermal regime: 94°C/10 min, 25x (94°C/60 s, 67°C/60 s, 72°C/30 s) and 72°C/10 min. Amplicons were resolved by agarose gel electrophoresis on a 1·5% agarose gel in TBE containing 2·5 μl ml^{-1} ethidium bromide and documented.

An oligonucleotide (5′-GCG TGC CGG GTT TAT GAC CT-3′), named BR1f, was designed from the IGS region of BPBB based on the restriction enzyme site of *Sex*AI, and used with the primer L1r to detect BPBB strains. PCR amplification was carried out as stated above, with 1 μM each of primer BR1f and L1r, with the following thermal regime: 94°C/2 min, 25x (94°C/45 s, 62°C/45 s, 72°C/90 s) and 72°C/10 min.

RESULTS

Biochemical and physiological tests

Selected phenotypic characteristics that differentiate BPBB from the other subspecies of *E. carotovora* and *E. chrysanthemi* are presented in Table 2. Strains of BPBB were pectolytic on CVP, produced acid from α-methyl-glucoside and reducing substances from sucrose, grew at 37°C, and did not utilize psicose, Tween-40 nor Tween-80 as sole carbon source. All strains of *E. c. atroseptica* tested utilized D-galactonic acid lactone, D-gluconic acid, uridine, and thymidine, while only 12–38% of BPBB strains did so. Moreover, none of the *E. c. atroseptica* strains utilized D-galacturonic acid, D-saccharic acid, or L-glutamic acid, while 69–75% of BPBB strains utilized these carbon sources.

The Biolog system identified all strains of *E. c. atroseptica*, *E. c. betavasculorum* and *E. chrysanthemi* correctly but BPBB, *E. c. odorifera* and *E. c. wasabiae* were identified as *E. c. carotovora*. Differential oxidation (given as percentage of BPBB strains positive/percentage of *E. c. carotovora* strains positive) of D,L-lactic acid (69/20), glucose-1-phosphate (0/60), L-lactic acid (94/0), α-methyl D-glucoside (81/0), palatinose (94/0), psicose (6/100), inosine (38/100),

Table 2 Selected phenotypic characteristics that differentiate Brazilian potato blackleg bacteria and five other subspecies of *E. carotovora* and *E. chrysanthemi*

Characteristic	Response of strains*						
	BPBB ($n = 16$)†	Eca ($n = 5$)	Ecc ($n = 5$)	Ecb ($n = 1$)	Eco ($n = 1$)	Ecw ($n = 1$)	Ech ($n = 1$)
Phosphatase	0	0	0	−	−	−	+
Acid from α-methyl glucoside	100	100	0	+	+	−	−
Reducing substances from sucrose	100	100	0	+	+	−	−
Growth at 37°C	100	0	100	+	+	−	+
ONPG Test	100	0	100	+	+	−	+
Erythromycin	0	0	0	−	−	−	+
Utilization of							
Acetic acid	50	25	80	+	−	+	+
Cellobiose	100	100	100	−	+	+	−
α-cyclodextrin	0	0	0	+	−	−	−
D-arabitol	0	0	0	+	+	−	−
2′-Deoxy adenosine	12	100	40	+	+	+	−
Dextrin	0	0	40	+	−	−	+
D-galactonic acid lactone	12	100	80	−	−	+	−
D-galacturonic acid	75	0	80	−	+	+	+
D-gluconic acid	19	100	80	+	−	+	+
D-glucosaminic acid	0	0	0	−	−	−	+
D-glucuronic acid	0	0	0	−	−	−	+
D,L-lactic acid	69	50	20	+	−	+	+
D-malic acid	88	20	0	+	−	−	+
D-melibiose	100	100	100	−	+	−	+
3-Methylglusose	0				+		
D-saccharic acid	75	0	80	+	−	−	+
D-sorbitol	0	0	20	+	−	−	+
D-trehalose	100	100	100	+	+	+	−
Gentiobiose	94	100	60	+	+	−	−
Glucose-1-phosphate	0	30	60	−	−	−	+
Inosine	38	50	100	+	+	+	−
Lactulose	6	80	0	−	−	−	−
L-glutamic acid	25	0	80	+	+	−	+
L-lactic acid	94	80	0	+	−	+	+
L-proline	0	0	0	+	−	−	−
Malonic acid	0	0	0	−	−	−	+
Maltose	19	25	0	+	+	−	+
N-acetyl-D-glucosamine	0	0	0	+	−	−	−
Palatinose	94	100	0	+	+	−	−
Psicose	6	40	100	+	+	+	+
Succinamic acid	100	0	100	+	+	+	+
Thymidine	38	100	100	+	+	+	+
Thymidine-5′-monophosphate	0				+		
Tween-40	0	0	20	+	−	−	−
Tween-80	0	0	60	+	−	−	+
Uridine	31	100	100	+	+	+	−

*BPBB, Brazilian potato blackleg bacteria – strains 8, 29, 54, 101, 106, 137, 138, 142, 153, 200, 201, 205, 212, 213, 219, and 371; Eca, *E. carotovora* subsp. *atroseptica* – strains 3, 6, 31, 196, and 198; Ecc, *E. carotovora* subsp. *carotovora* – strains 21, 51, 59, 63 and 193; Ecb, *E. carotovora* subsp. *betavasculorum* – strain LMG 2398; Eco, *E. carotovora* subsp. *odorifera* – strain 1878^T; Ecw, *E. carotovora* subsp. *wasabiae* – strain SR91^T; Ech, *E. chrysanthemi* – strain 571. Percentage of strains showing positive response or (−) negative and (+) positive results.
†Number of strains tested.

thymidine (38/100) and Tween-80 (0/60) revealed differences between BPBB and *E. c. carotovora* (Table 2).

Erwinia c. betavasculorum oxidized 22 carbon sources not metabolized by BPBB including α-cylcodextrin, D-arabitol, dextrin, D-sorbitol, L-proline, *N*-acetly-D-glucosamine, Tween-40 and Tween-80 (Table 2). But unlike *E. c. betavasculorum*, BPBB metabolized cellobiose and D-melibiose. Biochemically, BPBB strains were most similar to *E. c. odorifera*, but differed in being unable to utilize D-arabitol, 3-methylglucose and thymidine-5′-monophosphate (Table 2).

A dendrogram based on the numerical analysis, using the Euclidian distance coefficient and UPGMA, is represented in Fig. 1. *Erwinia c. betavasculorum* and *E. chrysanthemi* were distinct, but *E. c. odorifera*, *E. c. wasabiae* and *E. c. atroseptica* were closely related. The Euclidian distances among BPBB strains ranged from 0·139 to 0·275. Strains Ecc 193 and Ecc 59 were in the lower (0·137) and Ecc 51 in the higher range, showing that BPBB strains form an internal group in the heterogeneous and broader group of *E. c. carotovora* strains.

Pathogenicity and maceration activity

Potato stems inoculated with BPBB strains developed typical blackleg symptoms. However, symptoms of blackleg were visible 3 days after inoculation with BPBB in contrast to stems inoculated with *E. c. atroseptica* that required at least 7 days to develop symptoms. BPBB strains consistently macerated potato tuber tissue and green pepper fruits more rapidly than strains of *E. c. atroseptica* in three replications of the experiment (Fig. 2).

Serological specificity and fatty acid analyses

In the ELISA test with McAb 4F6, the mean absorbance value (A_{405}) for *E. c. atroseptica* strains was 0·287 ± 0·140 compared with 0·019 ± 0·010 for 149 BPBB strains and negative controls.

Fatty acid analysis showed that BPBB strains had no striking differences from the other *E. carotovora* subspecies as do *E. c. atroseptica* strains. The following six fatty acids, in order of decreasing amounts, were present in all the 46 strains of the six subspecies: hexadecanoid acid (16:0), octadecanoid acid (18:1 w7c), dodecanoid acid (12:0), tetradecanoid acid (14:0), pentadecanoid acid (15:0) and heptadecanoid acid (17:0). Also present in all subspecies but not in all strains were tridecanoid acid (13:0), and two unidentified fatty acids with equivalent chain lengths of 13·957 and 14·502 (Table 3). Along with *E. c. betavasculorum* and *E. c. odorifera*, BPBB had lower levels of tridecanoid

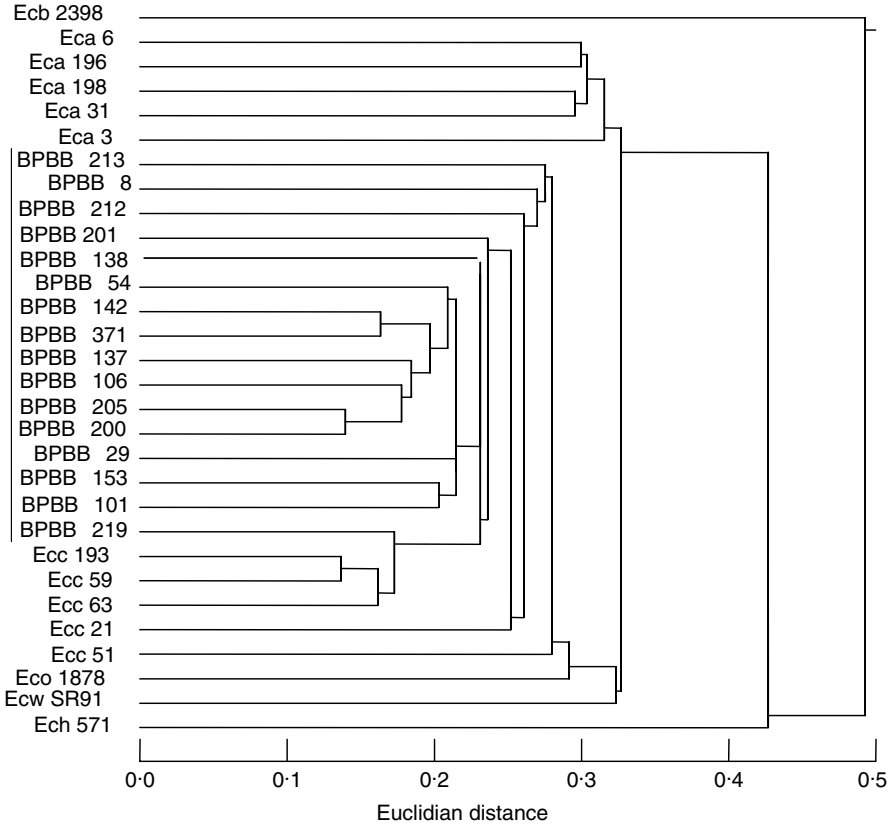

Fig. 1 Dendrogram based on the numerical analysis, using Euclidian distance coefficient and UPGMA, of 93 utilized carbon sources (borderline = 0·5; positive = 1) of the GN and GP MicroPlates (Biolog Inc.) by the Brazilian potato blackleg bacterium (BPBB), *E. carotovora* subsp. *atroseptica* (Eca), *E. carotovora* subsp. *betavasculorum* (Ecb), *E. carotovora* subsp. *carotovora* (Ecc), *E. carotovora* subsp. *odorifera* (Eco), *E. carotovora* subsp. *wasabiae* (Ecw) and *E. chrysanthemi* (Ech)

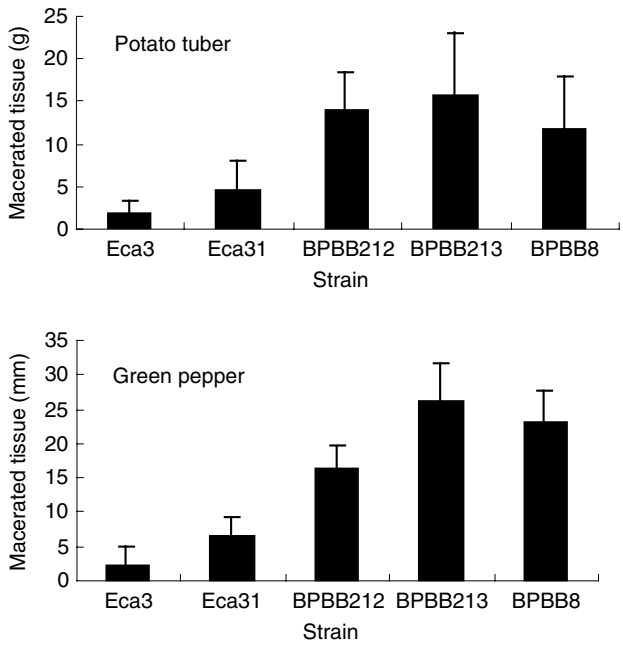

Fig. 2 Maceration activity of three strains of the Brazilian potato blackleg bacterium (BPBB) and two of *E. carotovora* subsp. *atroseptica* (Eca) on potato tubers and green peppers. Vertical lines show the standard deviation

acid (13:0) than *E. c. atroseptica*, *E. c. carotovora* and *E. c. wasabiae*. In two-dimensional cluster analysis using the FAME program, *E. c. atroseptica* strains clearly formed a separate cluster from the other subspecies and BPBB, none of which showed distinctly separated groups.

Sequencing and restriction analysis

Sequence data for the 16S rDNA region of BPBB strains 8, 212, 213 and 371 have been deposited in Genbank as accession numbers AY207086, AY207083, AY207084 and AY207085, respectively. 16S rDNA sequence analysis using different methods (UPGMA, neighbour-joining, minimum evolution, maximum parsimony) revealed a position for BPBB strains among the *E. carotovora* subspecies in the different trees (data not shown). The scores assigned in a BLAST search confirmed this relationship; the identity varied from 97 to 99% homology with *E. carotovora* and *E. chrysanthemi* strains.

PCR amplification of the ITS region generated two fragments (Fig. 3). A summary of percentage matches of global DNA alignment of ITS regions of BPBB strains against five other subspecies of *E. carotovora* and three other species of *Erwinia* are shown in Table 4. The sequence alignment of the small region showed the presence of a single copy of the $tRNA^{Glu}$ gene and a single copy of the $tRNA^{Ile}$ and $tRNA^{Ala}$ genes in the large region. PCR amplification of the ITS of BPBB strains yielded amplicons similar in size to strains of the *E. carotovora* subspecies but different from *E. chrysanthemi* (Table 4). Cleavage of these amplicons with restriction enzyme *Cfo*I produced DNA fragments for BPBB similar to *E. c. atroseptica*, *carotovora* and *odorifera* (Fig. 4). The differences among *E. c. betavasculorum*, *wasabiae* and *E. chrysanthemi* were clearly distinguished in the gel. Cleavage of the BPBB ITS region with the restriction enzyme *Rsa*I produced a pattern similar to *E. c. odorifera* (Fig. 4). The enzyme *Hpy*CH4III cleaved the ITS of all subspecies and *E. chrysanthemi* but not that of BPBB strains (Fig. 4). The *Sex*AI restriction site, starting at nucleotide position 122 in the small region of the BPBB ITS, was a unique nucleotide target (Fig. 5).

PCR amplification

None of the 16 strains of BPBB produced amplicons in PCR with the *E. c. atroseptica* primers in contrast to the single 690 bp amplicon obtained with all seven *E. c. atroseptica* strains tested (data not shown).

We observed amplified fragments using Y primers with 12 strains of BPBB but not with four (8, 212, 213 and 219) of the BPBB strains tested. No amplification of *E. c. atroseptica*, *betavasculorum* and *wasabiae* strains was obtained using these primers (data not shown).

An oligonucleotide (5′-GCG TGC CGG GTT TAT GAC CT-3′), named BR1f, was designed from the IGS region of BPBB, based on the *Sex*AI restriction enzyme site, and used with the primer L1r to amplify DNA from BPBB strains. By using these primers in PCR, a 322-bp fragment was amplified from DNA of all BPBB strains tested but no amplicons were obtained from DNA extracted from the five *E. carotovora* subspecies or *E. chrysanthemi* (Fig. 3).

DISCUSSION

Erwinia c. atroseptica is the major causal agent of potato blackleg in cool and temperate regions of Canada, the US and western Europe (Molina and Harrison 1977; Caron *et al.* 1979; Persson 1988; Bain *et al.* 1990). It can be differentiated from all other *E. carotovora* strains solely on the basis of acid production from α-methylglucoside, production of reducing substances from sucrose, and inability to grow at 37°C (Graham 1972; De Boer *et al.* 1979). The BPBB strains associated with blackleg in Brazil were clearly different from *E. c. atroseptica*. Although they produced reducing substances from sucrose and acid from α-methylglucoside, like *E. c. atroseptica*, they grew at 37°C, their DNA was not amplified in PCR with *E. c. atroseptica*-specific primers, and did not react with antibodies specific to the major *E. c. atroseptica* serogroup. Focusing on the

Table 3 Relative amount of fatty acids detected in various strains of the Brazilian potato blackleg bacterium and of strains in five subspecies of *Erwinia carotovora*

	BPBB* ($N = 14$)†		Eca ($N = 6$)		Ecb ($N = 3$)		Ecc ($N = 15$)		Eco ($N = 4$)		Ecw ($N = 4$)	
Fatty acid	Mean (%)	n‡	Mean (%)	n	Mean (%)	n	Mean (%)	n	Mean (%)	n	Mean (%)	n
12:0	6·45	14	5·73	6	6·66	3	6·76	15	6·45	4	6·00	4
13:0	0·39	13	0·57	3	0·37	2	0·88	7	0·31	3	0·61	4
12:0 3OH	–	0	–	0	0·46	3	–	0	–	0	–	0
Unknown 13·957	0·69	14	0·61	6	0·68	3	0·72	14	0·81	4	0·82	4
14:0	1·52	14	2·39	6	1·58	3	1·60	14	1·28	4	1·57	4
Unknown 14·502	1·09	14	0·93	5	1·01	3	1·17	13	1·00	4	0·77	2
15:0	1·30	14	2·16	6	0·87	3	1·75	15	0·72	4	2·08	4
16:0	27·25	14	33·57	6	29·10	3	27·72	15	26·34	4	29·46	4
17:0	1·18	14	0·89	4	0·78	3	1·45	15	0·90	4	1·24	4
17:1 w8c	0·50	12	0·68	2	0·40	2	0·89	13	0·53	4	0·57	4
17:0 w6c	–	0	–	0	–	0	0·63	4	–	0	–	0
17:0 cyclo	–	0	0·58	2	0·58	2	–	0	–	0	–	0
18:1 w7c	17·83	14	9·18	6	14·71	3	20·35	15	20·19	4	15·73	4
18:0	0·34	8	–	0	–	0	0·32	9	0·31	4	0·27	2
12:0/14:0	4·24		2·40		4·22		4·21		5·03		3·81	
16:0/12:0	4·22		5·86		4·37		4·10		4·09		4·91	

*BPBB, Brazilian potato blackleg bacterium – strains 8, 24, 29, 54, 101, 106, 137, 138, 142, 153, 212, 213, 219, and 371; Eca, *E. carotovora* subsp. *atroseptica* – strains 3, 6, 19, 31, 196, and 198; Ecb, *E. carotovora* subsp. *betavasculorum* – strains 2398, 2461 and 2464; Ecc, *E. carotovora* subsp. *carotovora* – strains 21, 23, 26, 51, 59, 61, 62, 63, 65, 67, 68, 71, 94, 193 and 194; Eco, *E. carotovora* subsp. *odorifera* – strains 1645, 1878, 1880 and 1893; Ecw, *E. carotovora* subsp. *wasabiae* – strains SR91^T, SR92, SR93 and SR94.

†N = number of strains tested.

‡n = number of strains with the fatty acid.

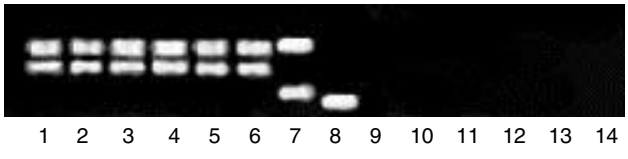

Fig. 3 PCR profiles of the 16S-23S intergenic spacer (IGS) regions of *Erwinia* sp. using primer set1491f/L1r (lanes 1–7), and primer set BR1f/L1r (lanes 8–14). Lanes 1 and 8, Brazilian potato blackleg bacterium BPBB 212; lanes 2 and 9, *E. carotovora* subsp. *atroseptica* Eca 3; lanes 3 and 10, *E. carotovora* subsp. *betavasculorum* Ecb 2398; lanes 4 and 11, *E. carotovora* subsp. *carotovora* Ecc 21; lanes 5 and 12, *E. carotovora* subsp. *odorifera* Eco 1878; lanes 6 and 13, *E. carotovora* subsp. *wasabiae* Ecw SR91; lanes 7 and 14, *E. chrysanthemi* Ech 571

production of reducing substances from sucrose and acid from α-methylglucoside as major traits, disregarding the ability to grow at 37°C, resulted in misidentification of *Erwinia* strains as *E. c. atroseptica* in the earlier study of Brazilian strains (de Oliveira 2001).

In addition to the above-mentioned biochemical and physiological features, BPBB strains also differed from *E. c. atroseptica* in their positive ONPG test for B-galactosidase and ability to utilize succinamic acid (Table 2). BPBB strains were similar to *E. c. betavasculorum* and *odorifera* in their ability to grow at 37°C, to produce acid from α-methylglucoside, and reducing substances from sucrose. However, they differed from *E. c. betavasculorum* and *E. c. odorifera* in their carbohydrate utilization patterns (Table 2).

Fatty acid analysis grouped the BPBB strains with *E. c. betavasculorum*, *carotovora*, *odorifera* and *wasabiae*, and distinguished them from *E. c. atroseptica*. Our results confirmed a previous study (De Boer and Sasser 1986) that differentiated *E. c. atroseptica* strains clearly as a separate cluster from the other subspecies, none of which show distinctly separated groups (Table 3). *Erwinia chrysanthemi* also presents its own characteristic fatty acid profile, lacking dodecanoid acid (De Boer and Sasser 1986). The usefulness of fatty acid profiles for characterizing soft rot erwinias was limited to identifying *E. c. atroseptica* and *E. chrysanthemi* and to differentiating them from the other subspecies of *E. carotovora*.

While the failure of PCR with the *E. c. atroseptica*-specific primers to amplify BPBB DNA has already been noted above, PCR amplification with the Y1/Y2 primer set targeting the *pel* genes was successful for DNA from 12 of 16 BPBB strains. Although we cannot readily explain the failure in amplification of the four BPBB strains other than

Table 4 Comparison of the nucleotide sequences of the small and large 16S-23S rRNA intergenic transcribed spacer (IGS) regions of several *Erwinia* spp. and subspp. to the Brazilian potato blackleg bacterium

		Small ITS region				Large ITS region			
*Species/ subspecies	Strain	NCBI accession	Length (bp)	Homology (%)	Matching nucleotides	NCBI accession†	Length (bp)	Homology (%)	Matching nucleotides
BPBB 212	ATCC BAA-417	AF448594	444	100	444	AF448595	484	100	484
BPBB 213	ATCC BAA-418	AF448596	451	94	427	AF448597	491	96	473
BPBBB 8	ATCC BAA-416	AF444592	452	94	425	AF448593	490	95	470
Eca	LMG 2386	AF232687	446	93	418	AF234282	475	87	435
Ecb		AF232686	445	94	422	AF234280	486	93	455
Ecc	ATCC 15713	AF232684	444	94	419	AF234284	487	93	457
Eco		AF232680	453	89	407	AF234278	489	93	458
Ecw	ATCC 43316	AF232679	448	93	417	AF234277	482	91	443
Ech	ATCC 11663	AF232681	356	59	267	AF234287	491	64	353
Eam		AF290419	726	43	316	AF290418	1031	35	366
Epy		AJ132969	422	55	283	–	–	–	–

*BPBB = Brazilian potato blackleg bacterium; Eca = *E. carotovora* subsp. *atroseptica*; Ecb = *E. carotovora* subsp. *betavasculorum*; Ecc = *E. carotovora* subsp. *carotovora*; ECC = *E. carotovora* subsp. *odorifera*; Ecw = *E. carotovora* subsp. *wasabiae*; Ech = *E. chrysanthemi*; Eam = *E. amylovora*; Epy = *E. pyrifoliae*.

†Sequences were retrieved from the GenBank (NCBI = National Center for Biotechnology Information) database under the accession numbers indicated.

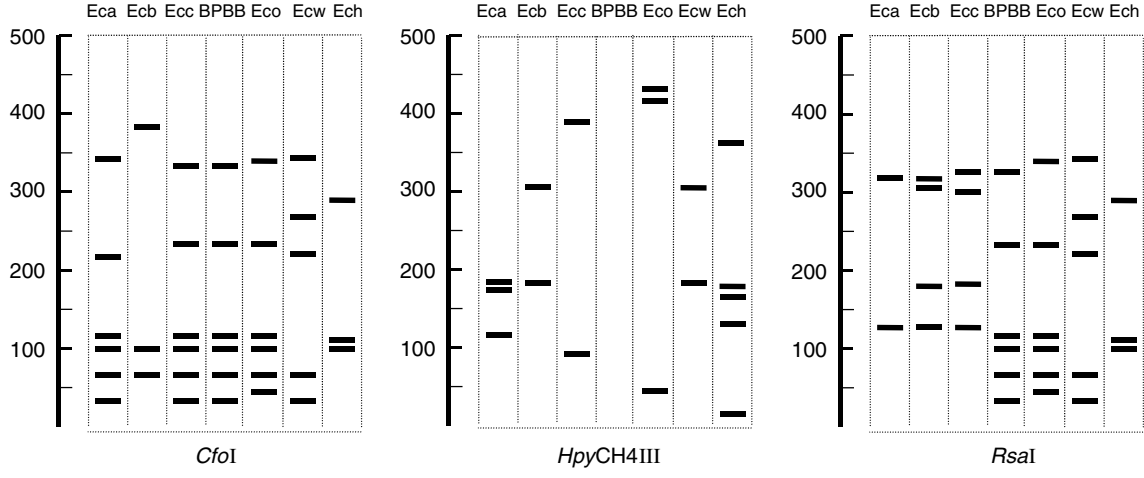

Fig. 4 Scheme representing gels of the *Cfo*I, *Hpy*CH4III and *Rsa*I restriction patterns of the 16S-23S rRNA regions of intergenic transcribed spacer (IGS) of the Brazilian potato blackleg bacterium (BPBB: AF448594, AF448595), *Erwinia carotovora* subsp. *atroseptica* (Eca: AF232687, AF234282), *E. carotovora* subsp. *betavasculorum* (Ecb: AF232686, AF234280), *E. carotovora* subsp. *carotovora* (Ecc: AF232684, AF234284), *E. carotovora* subsp. *odorifera* (Eco: AF232680, AF234278), *E. carotovora* subsp. *wasabiae* (Ecw: AF232679, AF234277), and *E. chrysanthemi* (Ech: AF232681, AF234287). Numbers denote accession codes of sequences of small and large IGS regions, respectively, retrieved from GenBank, National Center for Biotechnology Information database

to speculate on variation in the pectate genes, we note that DNA from several of our *E. c. atroseptica* strains also were not amplified with the Y1/Y2 primers. However, the failure of DNA from *E. c. betavasculourm* to be amplified in PCR with these primers is consistent with the published literature (Darrasse *et al.* 1994).

The 16S rDNA sequence of BPBB was consistent with its identity as a member of the *E. carotovora* species. BPBB strains were positioned among other strains of the species in dendrograms using various algorithms based on neighbour-joining, maximum parsimony, and unweighted pair-group methods of analysis. The sizes and sequences of the large

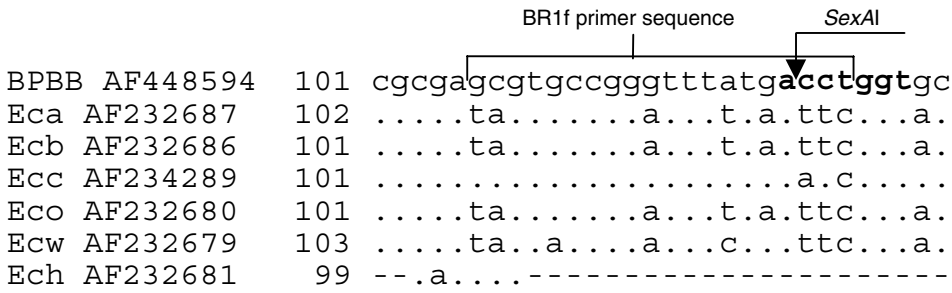

Fig. 5 Homology sequence alignment of the small 16S-23S rDNA intergenic transcribed spacer (IGS) region of Brazilian potato blackleg bacterium (BPBB), *E. carotovora* subsp. *atroseptica* (Eca), *E. carotovora* subsp. *betavasculorum* (Eca), *E. carotovora* subsp. *carotovora* (Ecc), *E. carotovora* subsp. *odorifera* (Eco), *E. carotovora* subsp. *wasabiae* (Ecw), and *E. chrysanthemi* (Ech) partially displayed to show the *Sex*AI restriction site (bold) present in BPBB and absent in the others, and the BR1f primer sequence. Sequences were retrieved from the GenBank (National Center for Biotechnology Information) database under the accession numbers indicated

and small IGS regions of BPBB strains were also similar to those of other *E. carotovora* subspecies and different from *E. chrysanthemi* (Fig. 3) (Fessehaie *et al.* 2002). All the *E. carotovora* subspecies could be differentiated by restriction fragment length polymorphisms in the IGS region following digestion with appropriate restriction enzymes (Fig. 4). The lack of a *Hpy*CH4III site in the IGS of BPBB strains is particularly noteworthy.

The unique *Sex*AI site in the small IGS region of BPBB strains was a useful target for designing a primer (BR1f) unique to these strains (Fig. 5). The PCR based on the BR1f primer did not amplify DNA from strains representing other *E. carotovora* subspecies or *E. chrysanthemi* (Fig. 3). PCR based on the BR1f primer is useful in determining the presence of BPBB-like strains on potato. In preliminary work, a multiplex PCR utilizing primer sets ECA1f/ECA2r and BR1f/L1r was used to show that BPBB-like strains, but not *E. c. atroseptica*, occur on ostensibly healthy tubers in Brazil while *E. c. atroseptica*, but not BPBB strains, occur on potato tubers in Canada.

The summation of our results strongly suggests that the BPBB strains form a new subspecies of *E. carotovora*. Although we lack resources for the DNA–DNA hybridization studies that are evidently required to establish new taxons of bacteria, it is not surprising that new subspecies will be identified among the heterogeneous and poorly defined *E. c. carotovora* strains. If a new subspecies were to be accepted for the BPBB strains, an appropriate subspecific epithet would be *E. carotovora* subsp. *brasiliensis*.

Although the BPBB strains occur in a geographical area that is considered a humid subtropical climate, the temperatures (17–20°C) during the growing season are relatively cool. The association of temperature with the occurrence of BPBB needs to be investigated. In any case, under the temperate conditions in which our pathogenicity and maceration tests were carried out, the BPBB strains were more virulent on potato than the *E. c. atroseptica* strains (Fig. 2). The high level of virulence shown by these strains, in fact, suggests that some circumspection may be appropriate to curtail their dissemination to other geographical areas.

ACKNOWLEDGEMENTS

We would like to express our gratitude to Roger Phillipe at the Centre for Plant Quarantine Pests, Ottawa, Canada, for the fatty acid analysis. We also recognize the importance of the scholarship given by CAPES – Brazilian Federal Agency – to Dr Valmir Duarte to spend his sabbatical working on this research.

REFERENCES

Bain, R.A., Perombelon, M.C.M., Tsror, L. and Nachmias, A. (1990) Blackleg development and tuber yield in relation to numbers of *Erwinia carotovora* subsp. *atroseptica* on seed potatoes. *Plant Pathology* **39**, 125–133.

Caron, M., Lachance, R.O. and Pelletier, G. (1979) Pathogenicite comparee d'*Erwinia carotvora* var. *atroseptica* et var. *carotovora* par inoculation de cultrivars de pomme de terre. *Canadian Journal of Plant Science* **59**, 411–416.

Darrasse, A., Priou, S., Kotoujansky, A. and Bertheau, Y. (1994) PCR and restriction fragment length polymorphism of a *pel* gene as a tool to identify *Erwinia carotovora* in relation to potato diseases. *Applied and Environmental Microbiology* **60**, 1437–1443.

De Boer, S.H. and Kelman, A. (2000) Gram-negative bacteria: *Erwinia* soft rot group. In *Laboratory Guide for Identification of Plant Pathogenic Bacteria*, 3rd edn. ed. Schaad, N.W., Jones, J.B. and Chun, W. pp. 56–72. St Paul, MN: American Phytopathological Society.

De Boer, S.H. and McNaughton, M.E. (1987) Monoclonal antibodies to the lipopolysaccharide of *Erwinia carotovora* subsp. *atroseptica* serogroup I. *Phytopathology* **77**, 828–832.

De Boer, S.H. and Sasser, M. (1986) Differentiation of *Erwinia carotovora* ssp. *carotovora* and *E. carotovora* ssp. *atroseptica* on the

basis of cellular fatty acid composition. *Canadian Journal of Microbiology* **32**, 796–800.

De Boer, S.H. and Ward, L.J. (1995) PCR detection of *Erwinia carotovora* subsp. *atroseptica* associated with potato tissue. *Phytopathology* **85**, 854–858.

De Boer, S.H., Copeman, R.J. and Vruggink, H. (1979) Serogroups of *Erwinia carotovora* potato strains determined with diffusible somatic antigens. *Phytopathology* **69**, 316–319.

De Boer, S.H., Verdonck, L., Vruggink, H., Bang, H.O. and De Ley, J. (1987) Serological and biochemical variation among potato strains of *Erwinia carotovora* subsp. *atroseptica* and their taxonomic relationship to other *E. carotovora* strains. *Journal of Applied Bacteriology* **63**, 487–495.

Fessehaie, A., De Boer, S.H. and Levesque, C.A. (2002) Molecular characterization of DNA encoding 16S-23S rRNA intergenic spacer regions and 16S rRNA of pectolytic *Erwinia* species. *Canadian Journal of Microbiology* **48**, 387–398.

Gallois, A., Samson, R., Ageron, E. and Gvozdyak, R.I. (1992) *Erwinia carotovora* subsp. *odorifera* subsp. nov., associated with odorous soft rot of chicory (*Cichorium intybus* L.). *International Journal of Systematic Bacteriology* **42**, 582–588.

Gardan, L., Couy, C., Christen, R. and Samson, R. (2003) Elevation of three subspecies of *Pectobacterium carotovorum* to species level: *Pectobacterium atrosepticum* sp. nov., *Pectobacterium betavasculorum* sp. nov. and *Pectobacterium wasabiae* sp. nov. *International Journal of Systematic and Evolutionary Microbiology* **53**, 381–391.

Goto, M. and Matsumoto, K. (1987) *Erwinia carotovora* subsp. *wasabiae* subsp. nov. isolated from diseased rhizomes and fibrous roots of Japanese horseradish. *International Journal of Systematic Bacteriology* **37**, 130–135.

Graham, D.C. (1972) Identification of soft-rot coliform bacteria. In *Proceedings of the 3rd International Conference on Plant Pathogenic Bacteria* ed. Maas-Geesteranus, H.P. pp. 273–279. Wageningen, Netherlands: Pudoc.

Hauben, L., Moore, E.R.B., Vauterin, L., Steenackers, M, Mergaert, J., Verdonck, L. and Swings, J. (1998) Phylogenetic position of phytopathogens with the Enterobacteriaceae. *Systematic and Applied Microbiology* **21**, 384–397.

Lee, S.H. and Cha, J.S. (2001) Efficient induction of bacterial soft rot using mineral oil. *Phytopathology* **91**, S53.

Molina, J.J. and Harrison, M.D. (1977) The role of *Erwinia carotovora* in the epidemiology of potato blackleg. I. Relationship of *E. carotovora* var. *carotovora* and *E. carotovora* var. *atroseptica* to potato blackleg in Colorado. *American Potato Journal* **54**, 587–591.

de Oliveira, A.M.R. (2001) Incidence and genetic variability of pectolytic erwinias associated with blackleg in potato fields in Rio Grande do Sul. *PhD thesis*, Universidade Federal do Rio Grande do Sul, Porto Alegre.

Pérombelon, M.C.M. (1992) Potato blackleg: epidemiology, host–pathogen interaction and control. *Netherlands Journal of Plant Pathology* **92**, 135–146.

Pérombelon, M.C.M. and Kelman, A. (1980) Ecology of the soft rot erwinias. *Annual Review of Phytopathology* **18**, 361–387.

Persson, P. (1988) Blackleg and stem rot of potatoes in Sweden. *Acta Agriculturae Scandinavica* **38**, 177–182.

Stackebrandt, E. & Goodfellow, M. (1991) *Nucleic Acid Techniques in Bacterial Systematics*. West Sussex, UK: John Wiley & Sons Ltd.

Thomson, S.V., Hildebrand, D.C. and Schroth, M.N. (1981) Identification and nutritional differentiation of the erwinia sugar beet pathogen from members of *Erwinia carotovora* and *Erwinia chrysanthemi*. *Phytopathology* **71**, 1037–1042.

Wilton, S.D., Lim, L., Dye, D. and Laing, N. (1997) Bandstab: a PCR-based alternative to cloning PCR products. *Biotechniques* **22**, 642–645.