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Genetic characterisation of *Pectobacterium wasabiae* causing soft rot disease of potato in New Zealand

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Abstract *Pectobacterium wasabiae* has a narrow host range, having previously only been associated with Japanese horseradish. However, recent characterisation of *Pectobacterium* causing soft rotting in New Zealand has identified putative *P. wasabiae* isolates pathogenic to potato. In this study, phylogenetic reconstruction of *acnA* and *mdh* DNA sequences and fluorescent amplified fragment length polymorphisms (fAFLP) were used to confirm the identity of the putative *P. wasabiae* isolates. Both methods clustered the potato isolates closely with the type strain for *P. wasabiae*, ICMP9121, and also differentiated them from other plant pathogenic enterobacteria. PCR, DNA hybridisation and hypersensitive response (HR) assays were subsequently used to investigate the presence in *P. wasabiae* of the type III secretion system (T3SS) as well as other virulence factors known to be involved in development of disease by enterobacteria. Although all *P. wasabiae* strains appeared to elicit a type III-dependent HR in tobacco, genes associated with the T3SS and the putative virulence factors HecB and DspE could not be detected. Thus, genetic characterisation of *P. wasabiae* confirmed that it is a naturally occurring pathogen on potato, which does not possess the same suite of virulence factors that are involved in the pathogenicity of other enterobacteria on this host.

Keywords *Erwinia* · *hrpN* · *Solanum tuberosum*

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Introduction

Pectobacterium carotovorum subsp. *carotovorum* (syn. *Erwinia carotovorum* subsp. *carotovorum*) and *Pectobacterium atrosepticum* (syn. *E. carotovorum* subsp. *atrosepticum*) are the primary enterobacteria responsible for soft rotting of potato in temperate climates. *Pectobacterium carotovorum* subsp. *carotovorum* has a broad host range, likely due to its ubiquity in natural environments (Avrova et al. 2002), whereas *P. atrosepticum* is found almost exclusively on potatoes, usually associated with an aerial stem disease called blackleg (Pérombelon 2002). More recently, the highly virulent enterobacteria *Pectobacterium carotovorum* subsp. *brasiliensis* (syn. *Erwinia carotovorum* subsp. *brasiliensis*) was found to cause soft rotting and blackleg on potato crops in Brazil. Genetic and biochemical analyses differentiated strains belonging to this subsp. from other *Pectobacterium* (Duarte et al. 2004). *Pectobacterium carotovorum* subsp. *brasiliensis* was subsequently isolated

from potatoes grown in other areas of the world (Ma et al. 2007; J. van der Waals, personal communication).

Pectobacterium was previously classified in the genus *Erwinia*, which was divided into numerous species and subsp. on the basis of molecular, biochemical and host range differences. Subsequent taxonomic studies led to the reclassification of many Erwinias and to the elevation of several subsp. to distinct species of *Pectobacterium* (Gardan et al. 2003). A number of the redefined species were originally isolated from hosts other than potato including *Pectobacterium betavasculorum* (Thomson et al. 1981), *Pectobacterium carotovorum* subsp. *oderiferum* (Gallois et al. 1992), and *P. wasabiae* (Goto and Matsumoto 1987), which cause diseases of sugar beet, chicory and Japanese horseradish, respectively. Another enterobacterial species, *Erwinia chrysanthemi*, is responsible for aerial soft rot and wilt on potato. Taxonomic revision divided *E. chrysanthemi* into six genomic species belonging to a novel genus, *Dickeya*. As a result, strains of *Dickeya* infecting potato were transferred to several of the six newly designated species (Samson et al. 2005).

The diversity of enterobacteria and their different host ranges imply that multiple mechanisms may be used to infect potato and other hosts. Secreted cell wall-degrading enzymes such as pectinases, cellulases and proteases that macerate plant tissue are the primary determinants of virulence (Thomson et al. 1999). However, very little is known about other factors involved in disease development or that confer host specificity. Genome sequencing of *P. atrosepticum* SCRI1043 uncovered six secretion systems that are likely to contribute to virulence, including a type III secretion system (T3SS) (Bell et al. 2004). The T3SS is encoded by *hrp* (hypersensitive response and pathogenicity) and *hrc* (hypersensitive response conserved) genes, which encode a molecular syringe that injects virulence (effector) proteins into host cells (Galán and Collmer 1999).

A functional T3SS has been identified in several strains of *Pectobacterium* and *Dickeya,* and the *hrp* genes have been shown to be important for virulence. In *P. atrosepticum*, mutations in *hrpN*, encoding a helper protein secreted via the T3SS, as well as structural genes *hrcC* and *hrcV* involved in the formation of the T3SS result in significantly reduced virulence on potato (Holeva et al. 2004). HrpN also produces a rapid hypersensitive response (HR) in a non-host, tobacco, indicating that the *hrp* genes may also be involved in host specificity (Yang et al. 2002). A disease-specific effector, DspE, secreted by the T3SS, has been shown to play a role in necrogenic disease development in *Erwinia amylovora* (Bogdanove et al. 1998) and in soft rot pathogenesis in *P. atrosepticum* (Holeva et al. 2004).

Type I secretion systems, like the alpha-haemolysin secretion system consisting of the secreted adhesion protein (HecA) and the associated activator (HecB), are present in several species of phytopathogenic enterobacteria, including *P. atrosepticum* and *Dickeya*. The role of these genes in the development of disease on potato has not been confirmed, but the *hec* cluster in *D. chrysanthemi* is linked to attachment, aggregation and epidermal cell killing in *Nicotiana* subsp. (Rojas et al. 2002).

Identification of *Pectobacterium* and *Dickeya* infecting potato relies predominantly on biochemical and genetic differentiation of isolates (e.g. De Boer and Sasser 1986; Toth et al. 2001). However, studies investigating the diversity of enterobacteria on potatoes have found numerous atypical strains that could not be classified using these diagnostic techniques (Oliveira et al. 2003; Yahiaoui-Zaidi et al. 2003). Amplified Fragment Length Poylmorphism (AFLP) analyses and multilocus sequence typing (MLST) have also demonstrated a greater diversity of pectolytic enterobacteria infecting potato than previously thought (Avrova et al. 2002; Yap et al. 2004).

Recently, we found a variety of atypical strains whilst using a polyphasic approach to study the diversity of enterobacteria causing soft rotting of potatoes in New Zealand. A number of strains, although identified as *P. carotovorum* subsp. *carotovorum* by carbon utilisation profiling, produced no PCR product with primers specific to *P. carotovorum* subsp. *carotovorum* and clustered closely with *P. wasabiae* in phylogenetic analyses using the 16S rRNA gene (Pitman et al. 2008). Several studies have demonstrated that the 16S rRNA gene provides only coarse resolution of enterobacterial phytopathogens due to relatively conserved rates of mutation (Young and Park 2007). As a result, we have further characterised the atypical *P. wasabiae* strains isolated from potato tubers using AFLPs and phylogenetic analyses of *mdh* and *acnA*, as both methods have previously been shown to robustly differentiate strains of *Pectobacterium* and *Dickeya*.

We also investigated the presence of key virulence genes in enterobacteria that have been associated with disease development in potatoes in order to establish the possible mechanisms used by *P. wasabiae* to infect potato. We present new evidence confirming the identity of *P. wasabiae* isolated from potato tubers in New Zealand and demonstrate that these strains may use novel mechanisms for pathogenicity on potato.

Materials and methods

Bacterial strains and media

The bacterial strains used in this study were isolated from potatoes showing soft rot symptoms in New Zealand (Pitman et al. 2008) or obtained from the International Collection of Micro-organisms from Plants (ICMP), Landcare Research, New Zealand (Table 1). All bacterial strains were routinely grown on Luria-Bertani medium (LB) at 28°C for 12–24 h and kept at −80°C for storage.

DNA sequencing and multilocus sequence analyses

Genomic DNA was extracted from overnight broth cultures using a DNeasy tissue kit (Qiagen). For each bacterial strain, partial *acnA* and *mdh* DNA sequences (approximately 500 bp) were then amplified as they have previously been shown to be phylogenetically informative at this taxonomic level (Yap et al. 2004). PCR reactions were performed in a total volume of 25 µl consisting of 10 ng of template DNA, 0.2 U of *Taq* polymerase (Roche Diagnostics), 2.5 µl of 10× PCR buffer, 1.5 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphate (dNTP), and 0.2 µmol of each primer. PCR amplifications were carried out in a GeneAmp^R PCR System 9700 thermocycler (Applied Biosciences) with the following steps: (i) initial denaturation at 95°C for 5 min; (ii) 35 amplification cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 60 s; and (iii) a terminal extension phase at 72°C for 7 min. Primers used to amplify the *acnA* and *mdh* genes are listed in Table 2.

PCR fragments were purified with a QIAquick PCR purification kit (Qiagen), and were sequenced using an Automatic Sequencer 3730× (Macrogen Inc., Korea). DNA sequences were then assembled and edited with Sequencher 4.5 (Gene Codes), and deposited in GenBank (Table 1). Partial *acnA* and *mdh* gene sequences were aligned using ClustalX 1.83 (Thompson et al. 1997) and both ends of the alignments were trimmed to produce the following DNA sequences: *acnA*, 412 positions; *mdh*, 435 positions. Partial 16S rRNA gene sequences for each strain used in this study were obtained from previously deposited GenBank accessions (Pitman et al. 2008). For phylogenetic comparison, partial *mdh* DNA sequences from enterobacteria associated with soft rot of potato overseas were also retrieved from the GenBank database (Table 1).

Phylogenetic analysis of aligned *acnA*, *mdh* and 16S rRNA gene sequences was completed using PAUP* (edition 4.0b10) (Swofford 2002). Firstly, the three individual data sets were compared statistically for incongruence using the partition homogeneity test. Neighbor-joining and Maximum parsimony analyses were then performed on the individual and combined data sets incorporating 21 taxa using Kimura 2-parameter distances and the heuristic search option, respectively. Published sequences of *Yersinia pestis* and *Y. pseudotuberculosis* were used to root the resulting gene trees by the outgroup method (Ma et al. 2007). Stability of clades was assessed by 1,000 parsimony bootstrap replicates implemented in PAUP.

Phylogenetic relationships between the partial *mdh* gene sequences obtained from isolates on potato in this study and those previously submitted to the GenBank database were analysed using PAUP. DNA sequences, representing 29 taxa, were aligned using ClustalX 1.83 and then trimmed to 313 characters (due to differences in the lengths of GenBank submissions). Uninformative characters were excluded from the dataset before Maximum likelihood and Maximum parsimony analyses were performed using the heuristic search option. *Dickeya* sp. NZEC151 was selected as the outgroup for rooting of the Maximum likelihood tree.

Fluorescent Amplified Length Polymorphism (fAFLP) analysis

Fluorescent AFLP analysis was performed as previously described (Vos et al. 1995), with minor modifications. Genomic DNA (750 ng) was digested with *Eco*RI and *Mse*I prior to ligation with *Eco*RI and *Mse*I double-stranded adaptors in a total volume of

Table 1 Bacterial strains used in this study

Strain	Host	Geographical origin	Accession number		Reference/source
			acnA	*mdh*	
P. carotovorum subsp. *carotovorum*					
ICMP5702^T	*Solanum tuberosum*	Denmark	GQ144392	GQ144372	ICMP
NZEC23	*S. tuberosum*	New Zealand	GQ144398	GQ144378	Pitman et al. (2008)
NZEC115	*S. tuberosum*	New Zealand	N/D	N/D	Pitman et al. (2008)
NZEC118	*S. tuberosum*	New Zealand	GQ144404	GQ144384	Pitman et al. (2008)
NZEC143	*S. tuberosum*	New Zealand	N/D	N/D	Pitman et al. (2008)
WPP220	*Tagetes patula*	USA	N/F	*EF550798*	Ma et al. (2007)
WPP236	*Arctium minus*	USA	N/F	*EF550808*	Ma et al. (2007)
WPP17	*S. tuberosum*	USA	N/F	*EF550759*	Yap et al. (2004)
WPP14	*S. tuberosum*	USA	N/F	*EF550761*	Yap et al. (2004)
Ecc21	*S. tuberosum*	Netherlands	N/F	*EF550779*	De Boer and Sasser (1986)
Ecc193	*S. tuberosum*	USA	N/F	*EF550782*	De Boer and Sasser (1986)
P. carotovorum subsp. *oderiferum*					
ICMP11533^T	*Chicorium intybus*	France	GQ144390	GQ144370	ICMP
P. carotovorum subsp. *brasiliensis*					
BAA-417	*S. tuberosum*	Brazil	N/F	*EF550786*	Duarte et al. (2004)
BAA-419	*S. tuberosum*	Brazil	N/F	*EF550787*	Duarte et al. (2004)
WPP165	*S. tuberosum*	USA	N/F	*EF550795*	Ma et al. (2007)
P. atrosepticum					
ICMP1526^T	*S. tuberosum*	UK	GQ144393	GQ144373	ICMP
ICMP11299	*S. tuberosum*	New Zealand	N/D	N/D	ICMP
NZEC20	*S. tuberosum*	New Zealand	GQ144394	GQ144374	Pitman et al. (2008)
Eca6	*S. tuberosum*	USA	N/F	*EF550783*	De Boer and Sasser (1986)
SCRI1043	*S. tuberosum*	UK	N/F	*EF550788*	De Boer and Sasser (1986)
P. betavasculorum					
ICMP4226^T	*Beta vulgaris*	USA	GQ144389	GQ144369	ICMP
P. wasabiae					
ICMP9121^T	*Eutrema wasabi*	Japan	GQ144391	GQ144371	ICMP
NZEC9	*S. tuberosum*	New Zealand	GQ144395	GQ144375	Pitman et al. (2008)
NZEC10	*S. tuberosum*	New Zealand	GQ144396	GQ144376	Pitman et al. (2008)
NZEC12	*S. tuberosum*	New Zealand	GQ144397	GQ144377	Pitman et al. (2008)
NZEC8974	*S. tuberosum*	New Zealand	GQ144388	GQ144368	Pitman et al. (2008)
WPP19	*S. tuberosum*	USA	N/F	*EF550760*	Yap et al. (2004)
WPP161	*S. tuberosum*	USA	N/F	*EF550793*	Ma et al. (2007)
WPP163	*S. tuberosum*	USA	N/F	*EF550794*	Ma et al. (2007)
WPP168	*S. tuberosum*	USA	N/F	*EF550796*	Ma et al. (2007)
WPP172	*S. tuberosum*	USA	N/F	*EF550809*	Ma et al. (2007)
Pectobacterium spp.					
NZEC210	*S. tuberosum*	New Zealand	GQ144386	GQ144366	Pitman et al. (2008)
NZEC211	*S. tuberosum*	New Zealand	GQ144387	GQ144367	Pitman et al. (2008)
Dickeya spp.					
ICMP4649	*Daucus carota*	New Zealand	GQ144402	GQ144382	ICMP
ICMP9288	*S. tuberosum*	Papua New Guinea	GQ144401	GQ144381	ICMP

Table 1 (continued)

Strain	Host	Geographical origin	Accession number		Reference/source
			acnA	*mdh*	
ICMP9290	*Ipomoea batatas*	Papua New Guinea	GQ144400	GQ144380	ICMP
NZEC127	*S. tuberosum*	New Zealand	GQ144403	GQ144383	Pitman et al. (2008)
NZEC135	*S. tuberosum*	New Zealand	GQ144405	GQ144385	Pitman et al. (2008)
NZEC151	*S. tuberosum*	New Zealand	GQ144399	GQ144379	Pitman et al. (2008)

N/F, not found in GenBank; N/D, not determined; ^{T}, Type strain

Accession numbers in plain text were derived in this study. Those in italics were obtained from GenBank.

35 µl for 3 h at 37°C. The DNA was then amplified by PCR using the non-selective *Eco*RI and *Mse*I primers, E00 and M00, respectively. Each 25 µl reaction contained 2 µl of the ligation mixture, 1 U of AmpliTaq, 200 µM of each dNTP, 1.5 mM $MgCl_2$, and 50 ng each of the *Eco*RI and *Mse*I primers. PCR reactions were performed using 35 cycles of 30 s denaturing at 94°C, 30 s annealing at 60°C, and 1 min of extension at 72°C, followed by a final extension step of 72°C for 20 min. Following a 3-fold dilution, 2 µl of the non-selective PCR amplification product was used as template for a selective PCR with primers E00 and MseI-C. For selective PCR, the 11 µl reaction mixture contained 15 ng of the E00 primer, 3.5 ng of the MseI-C primer, 1.5 mM $MgCl_2$, 200 µM dNTPs and 1 U of Taq polymerase. Thermal cycling was performed using: (i) 12 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 1 min, decreasing the annealing temperature by 1.4°C every 2 cycles; and (ii) 23 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min with a final extension at 72°C for 40 min. Finally, AFLP products were visualised by a PRISM 310 Genetic Analyser (Applied Biosystems) using the GeneScan 500 size standard (Applied Biosystems). All primers used in the fAFLP reactions are listed in Table 2.

Table 2 Primers used in this study

Primer	Sequence (5′ to 3′)
mdh2	GCGCGTAAGCCGGGTATGGA
mdh4	CGCGGCAGCCTGGCCCATAG
acnA1	GCCTCGCCGCCGCTGGTGGT
acnA2	CCGCGCATCATCACTTCATG
E00	GACTGCGTACCAATTC
M00	GATGAGTCCTGAGTAA
MseI-C	GATGAGTCCTGAGTAAC
hrpN_F1	CTTCTTTGCAAATYACGATCAA
hrpN_R2	CGCYTTRGCCCAGGATTT
DSPE1F	GCAGCAAAACGCCCTGA
DSPE1R	GGTATTGAACCCTTCGCTCTTC
hecB_F6	GARCAGGGCATGGAACA
hecB_R5	TRAARKYRTTGCGGTAACGACTTT

Y (CT), R (AG), K (GT)

AFLP profiles were sized using GeneMarker (version 1.0.5.0, SoftGenetics LLC). A binary table was then obtained by scoring amplified fragments as present (1) or absent (0) and the data extrapolated to produce a similarity matrix using the Jaccard coefficient (Jc_{ij}) (Jaccard 1908). Finally, the data set of AFLP coefficients was used to construct a dendrogram by unweighted pair group method with arithmetic mean (UPMGA) cluster analysis using the MultiVariate Statistical Package (Kovach Computing Services).

Identification of enterobacterial virulence factors

Initially, degenerate primers (Table 2) were designed for PCR amplification of the *hrpN*, *dspE* and *hecB* genes using the consensus sequences from *P. atrosepticum* and *P. carotovorum* subsp. *carotovorum*. PCR reactions were then performed in a total volume of 25 µl consisting of 10 ng of template DNA, 0.2 U of *Taq* polymerase (Roche Diagnostics), 2.5 µl of 10× PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, and 0.2 µmol of each primer. Each reaction was carried out as follows: initial denaturation at 94°C for 5 min; two cycles of 20 s denaturing at 94°C, 20 s annealing at 60°C, and 2 min of extension at 72°C; two cycles

of 20 s denaturing at 94°C, 20 s annealing at 57°C, and 2 min of extension at 72°C; two cycles of 20 s denaturing at 94°C, 20 s annealing at 55°C, and 2 min of extension at 72°C; 30 cycles of 20 s denaturing at 94°C, 20 s annealing at 50°C, and 2 min of extension at 72°C. Reactions were terminated by a final extension step at 72°C for 7 min. Amplicons of 771 bp, 1,120 bp and 361 bp were expected for *hrpN*, *dspE* and *hecB*, respectively.

Southern hybridisations were performed using the recommended procedure of Roche Diagnostics. Firstly, the primers for *hrpN*, *dspE* and *hecB* were used to amplify three Digoxygenin (DIG)—labelled probes from *P. atrosepticum* strain ICMP1526 using the PCR DIG Probe Synthesis Kit (Roche). *Bgl*II-digested chromosomal DNA from selected enterobacteria was then separated by gel electrophoresis, transferred to Hybond nylon membranes (Amersham) and hybridised with one of three DIG-labelled probes. After hybridisation the membranes were washed at low stringency in a 2×SSC, 0.1% SDS solution.

The partial nucleotide sequences of the *hecB* gene amplified from different isolates of *Pectobacterium* in this study were deposited in GenBank under the accession numbers GQ144361 to GQ144365.

HR assays

HR assays (four replicates) were performed using tobacco leaves (*Nicotiana tabacum* cv. `Xanthi'). Mid-log-phase cultures of enterobacterial strains were washed twice with sterile deionised water. The first fully expanded tobacco leaves were then infiltrated with a suspension containing 2×10^8 CFU of bacteria ml^{-1} and the inoculated plants incubated at room temperature for 24–48 h.

Results

Phylogenetic characterisation of *P. wasabiae* on potato

Partial DNA sequences were obtained for the *acnA*, *mdh* and 16S rRNA genes of *Pectobacterium* causing soft rot of potatoes in New Zealand as well as a selection of type strains and other well characterised isolates of soft rotting enterobacteria. Given the recorded fragility of the 16S rDNA sequence for phylogenetic classification of *Pectobacterium*, the congruence of the three genes was evaluated via a partition homogeneity test. The test revealed significant incongruence ($P=0.01$), suggesting that it would be inappropriate to combine the datasets for the purposes of phylogenetic reconstruction. However, removal of the 16S rDNA sequence data identified significant homogeneity between *acnA* and *mdh* ($P=0.22$) as previously shown by Yap et al. (2004). Based on the results of the partition homogeneity test and the accepted use of *acnA* and *mdh* in other studies of enterobacteria, sequence data from *acnA* and *mdh* were combined for the phylogenetic analyses of putative *P. wasabiae* strains.

Phylogenetic reconstruction using the concatenated *acnA* and *mdh* DNA sequences resolved the relationships between New Zealand isolates and other well characterised strains of soft rotting enterobacteria, dividing the *Pectobacterium* into four distinct and strongly supported clades (Fig. 1). All strains previously identified as *P. carotovorum* subsp. *carotovorum* clustered into a single clade (clade B) supported by a bootstrap value of 89%. Clade B was represented by type strain *P. carotovorum* subsp. *carotovorum* ICMP5702, but also included the type strain for *P. carotovorum* subsp. *oderiferum,* confirming its classification as a subsp. of *P. carotovorum* in other studies (Ma et al. 2007). Several isolates from potato tubers clustered closely with the type strain for *P. wasabiae*, ICMP9121, in Clade C, corroborating previous research that showed the presence of *P. wasabiae* strains infecting potato in New Zealand (Pitman et al. 2008). This relationship was supported by a 92% bootstrap value. The phylogenies also confirmed the existence of a novel group of strains, distinct, but related to the known species of phytopathogenic *Pectobacterium*. These strains, which previously formed a unique clade using 16S DNA sequencing, clustered within a separate clade (Clade A) using the *acnA* and *mdh* genes and are represented by the highly virulent strain NZEC211.

The partial *mdh* DNA sequences from the New Zealand isolates obtained in this study were also compared to the *mdh* gene sequences from other closely related enterobacteria previously found on potato overseas (obtained from GenBank) using Maximum likelihood. Phylogenetic reconstruction grouped the isolates into four clades (A–D) (Fig. 2),

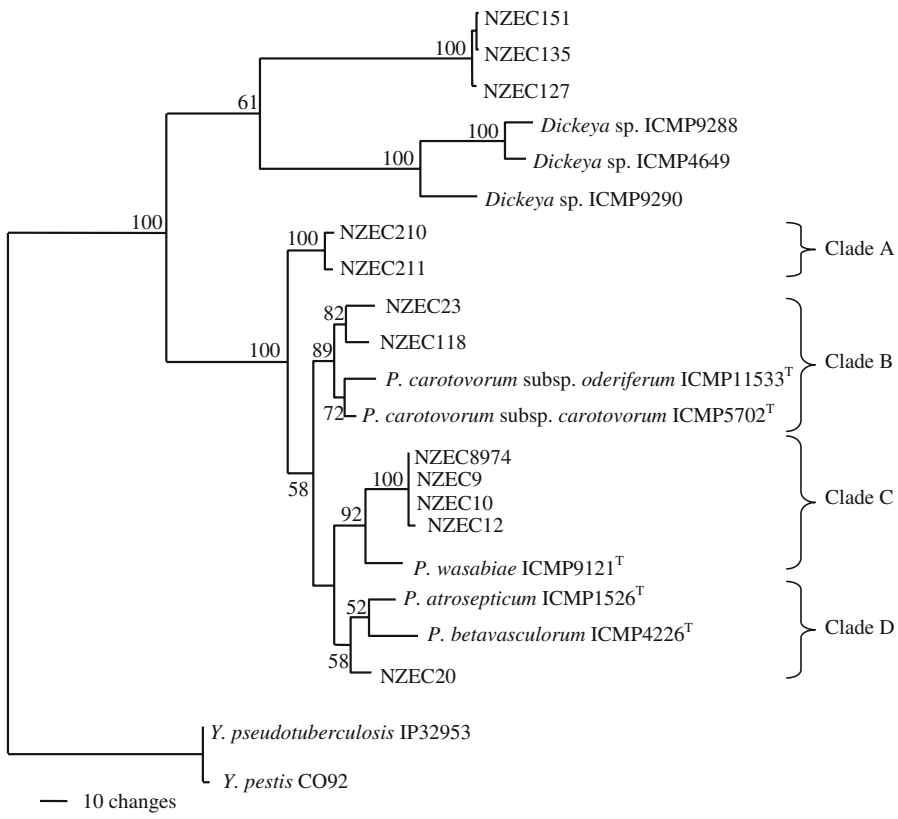

Fig. 1 A Maximum parsimony phylogram showing the relationships between putative *P. wasabiae* isolates from New Zealand and other enterobacterial strains based on concatenated *acnA* and *mdh* DNA sequences (tree 1 of 8 equally parsimonious trees produced using heuristic searches). Numbers by nodes represent bootstrap support >50% from 1,000 replicates. ^{T}, Type strain

similar to those found using the concatenated *acnA* and *mdh* DNA sequences. The New Zealand *P. wasabiae* strains clustered together, and with several isolates from the USA that had tentatively been described as *P. wasabiae* in previous studies (Ma et al. 2007). Bootstrap support for this clade (Clade C) was 81%, discriminating this group of isolates from *P. atrosepticum* (Clade D) and *P. carotovorum* subsp. *carotovorum* (Clade B). The *P. wasabiae* isolates were also distinguished from representative strains of *P. carotovorum* subsp. *brasiliensis*, which clustered together within Clade B, consistent with their status as a subsp. of *P. carotovorum*.

Cluster analyses using fAFLPs confirms the identity of *P. wasabiae* strains infecting potato in New Zealand

The identity of putative *P. wasabiae* strains causing soft rot of potatoes in New Zealand was confirmed by comparison with a selection of type strains and other well characterised strains of soft rotting *Pectobacterium* using fAFLP fingerprinting. The restriction enzymes *Eco*RI and *Mse*I, together with the selective primers E00 and MseI-C generated 174 diverse fAFLP peaks in the set of 17 *Pectobacterium* strains within a range of 40–550 bp. Analysis of the resulting Jaccard coefficients identified four distinct clusters at the Jc_{ij} level of 0.26 (Fig. 3), grouping the strains into the four clades (A–D) resolved using phylogenetic construction of the *acnA* and *mdh* DNA sequences.

Cluster B included all the strains previously identified as *P. carotovorum* subsp. *carotovorum* using biochemical tests and 16S rDNA sequencing (Pitman et al. 2008). AFLP fingerprinting also aligned *P. carotovorum* subsp. *oderiferum* into cluster B, congruent with its status as a subsp. of *P. carotovorum*. Cluster C contained all the putative *P. wasabiae* strains from New Zealand. These strains were linked together at a Jc_{ij} level of 0.70 and with the type strain for *P. wasabiae*, ICMP9121, at a Jc_{ij} level of 0.4. According to the AFLP results, this cluster was the most distantly related of any of the clusters containing known species of *Pectobacterium*.

At the Jc_{ij} level of 0.26, strains of *P. atrosepticum* and *P. betavasculorum* grouped together in cluster D,

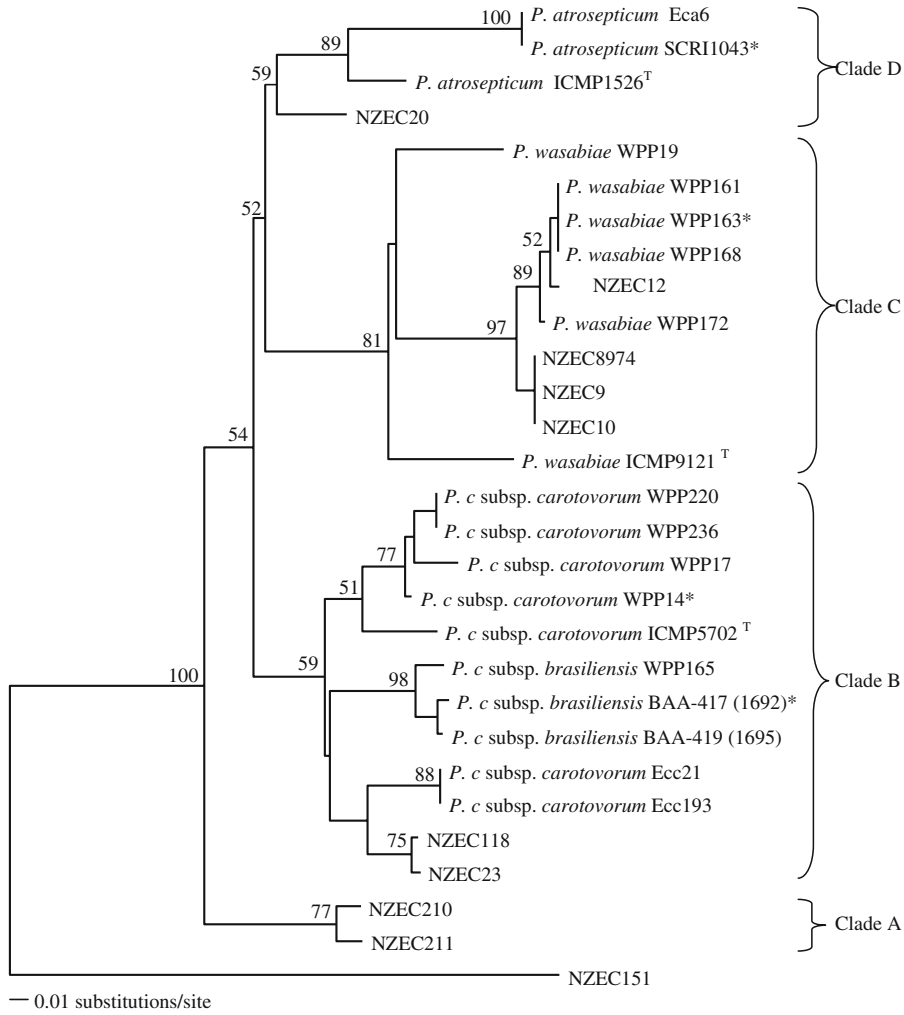

Fig. 2 Maximum likelihood tree showing relationships of *P. wasabiae* collected from potatoes and closely related enterobacteria isolated from potato overseas based on comparative analysis of *mdh* DNA sequences. Numbers by nodes represent bootstrap support >50% from 1,000 replicates using heuristic searches. ^{T}, Type strain. *, Strains for which genome sequencing is publically available

similar to the results from phylogenetic analyses of *acnA* and *mdh* DNA sequences. However, these isolates could be resolved further at a Jc_{ij} level of 0.27 using fAFLPs, indicating that *P. atrosepticum* and *P. betavasculorum* are genetically distinct. Cluster A contained several New Zealand isolates different from all other known *Pectobacterium* in this study. Their alignment into a unique fAFLP cluster confirmed the results of the phylogenetic studies using the *acnA* and *mdh* genes.

Divergence of the T3SS and key virulence factors in *Pectobacterium*

PCR amplification of *hrpN*, *dspE*, and *hecB* produced DNA fragments of 771, 1,120 and 361 bp, respectively, in *P. atrosepticum*, *P. carotovorum* subsp. *carotovorum* and *P. carotovorum* subsp. *odoriferum* (Fig. 4). The *hrpN* and *dspE* genes were also amplified from *P. betavasculorum*. However, with the exception of NZEC12, all the *P. wasabiae* strains failed to produce a PCR product indicative of *hrpN*, *dspE*, or *hecB*, further differentiating the *P. wasabiae* strains found on potatoes from other closely related *Pectobacterium*. Southern blots, probed with a DNA fragment containing *hrpN*, *dspE* or *hecB,* confirmed that the virulence-associated genes were absent or highly divergent in *P. wasabiae*. As shown in Fig. 4, the *hrpN*, *dspE*, and *hecB* probes only hybridised to the chromosomal DNA of strains that could also be used to amplify the corresponding genes by PCR.

PCR and Southern hybridisation showed the presence of one of the virulence-associated genes

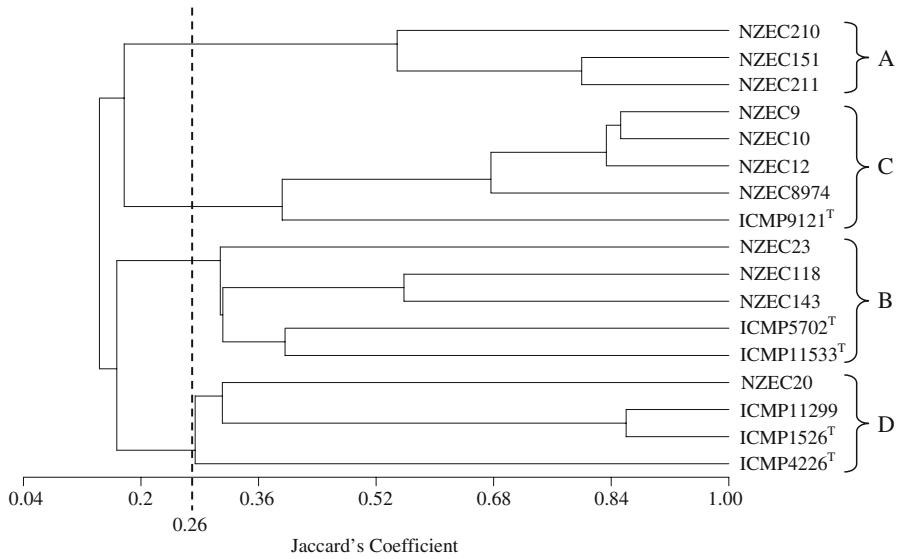

Fig. 3 A dendrogram constructed using UPGMA linkage of Jaccard's coefficients derived from fAFLP banding patterns of *Pectobacterium*. A Jc_{ij}=0.26 cut off value was used to divide the species of *Pectobacterium* into four distinct clusters. ^{T}, Type strain

tested in this study, *hecB*, in NZEC12. A partial *hecB* fragment of 321 bp was subsequently amplified from *P. wasabiae* NZEC12, *P. atrosepticum* ICMP1526, *P. carotovorum* subsp. *carotovorum* ICMP5702 and *P. carotovorum* subsp. *odoriferum* ICMP11533 using the degenerate primers hecB_F6 and hecB_R5 (Table 2). Cluster analysis of the resulting *hecB* DNA sequences showed that *hecB* from NZEC12 was divergent from those found in both *P. atrosepticum* and *P. carotovorum* (data not shown).

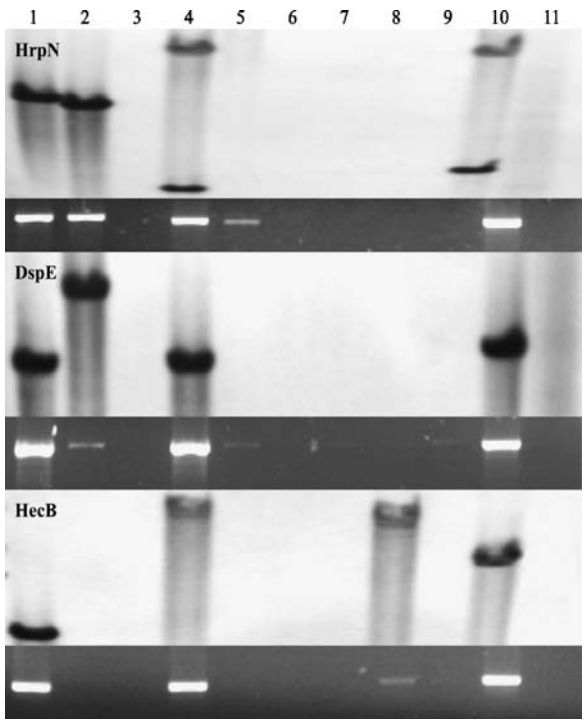

Fig. 4 Detection of *hrpN*, *dspE* and *hecB* in various *Pectobacterium* using Southern hybridisation and gene-specific PCR. Lanes 1–10, ICMP1526, ICMP4226, ICMP9121, ICMP5702, ICMP9290, NZEC9, NZEC10, NZEC12, NZEC8974, and ICMP11533, respectively

Pectobacterium wasabiae causes a mild HR response on tobacco

Infiltration of tobacco leaves with all strains of *P. wasabiae* used in this study resulted in the collapse of the plant cells at the point of infection, although NZEC10 and NZEC9 only produced a weak response (Fig. 5). The collapse of the plant tissue, characteristic of a HR, indicated that *P. wasabiae* strains infecting potato in New Zealand possess a functional T3SS and HrpN.

Discussion

The identity of *P. wasabiae* causing soft rotting of potato in New Zealand was confirmed by phylogenetic analyses of the *acnA* and *mdh* genes from a variety of phytopathogenic enterobacteria. All putative *P. wasabiae* isolates clustered closely together, along with the *P. wasabiae* type strain, ICMP9121, using a comparison of concatenated *acnA* and *mdh* DNA sequences. Phylogenetic analyses of the *mdh*

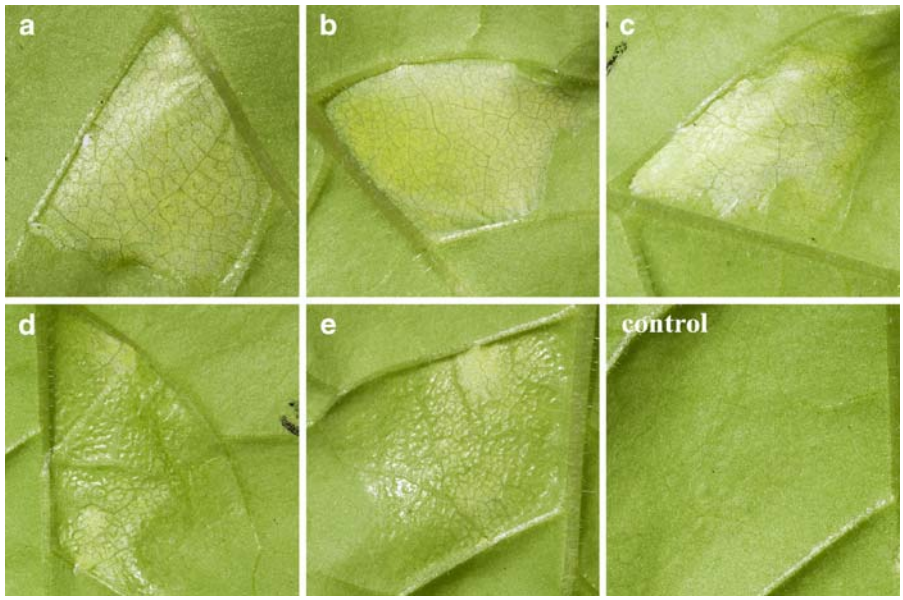

Fig. 5 The HR in tobacco leaves upon inoculation with *P. wasabiae* strains **a** ICMP9121, **b** NZEC8974, **c** NZEC12, **d** NZEC9, and **e** NZEC10. Sterile deionised water was used as a negative control

DNA sequences by Maximum likelihood also clustered these isolates within the same strongly supported clade as strains collected from potato in the USA that were tentatively described as *P. wasabiae* using DNA fingerprinting and MLST (Yap et al. 2004; Ma et al. 2007). Yap et al. (2004) found isolates of *P. wasabiae* and other species of *Pectobacterium* collected from different plant hosts grouped together, suggesting that these species had a wide host range. The results of our study provide further evidence that *P. wasabiae*, which had previously been thought to be host-specific, has a broad host range similar to *P. carotovorum*.

Although the trees generated by the *acnA* and *mdh* genes were not entirely congruent with those created using partial 16S rDNA sequences, the major clades resolved in this phylogenetic study were in accordance with our previous results using the 16S rRNA gene (Pitman et al. 2008). *Pectobacterium wasabiae* (Clade C) was strongly differentiated from all the known *Pectobacterium* responsible for diseases on potato, including *P. atrosepticum* (Clade D), *P. carotovorum* subsp. *carotovorum* and *P. carotovorum* subsp. *brasiliensis* (Clade B). However, the relationships among several of these species varied from those observed using the 16S rRNA gene. In other studies, comparative analyses of 16S rDNA sequences from *Pectobacterium* have demonstrated that this gene only provides coarse resolution of phytopathogenic enterobacteria (Young and Park 2007) or that this gene may be relatively unrepresentative of the bacterial genome (Zeigler 2003). In contrast, the *mdh* gene has been used successfully to compare the relationships among enteric bacteria (e.g. Boyd et al. 1994). Ma et al. (2007) used *mdh* as well as *acnA*, *gapA*, *icdA*, *mtlD*, *pgi* and *proA* for multilocus sequence analysis of enterobacteria. As in our study using *acnA* and *mdh*, these genes produced congruent trees emphasising the importance of choosing genes representative of the bacterial genome and the benefit of a multilocus approach for classification of enterobacteria.

An alternative method used for investigating diversity is AFLP analysis—one of the most sensitive genomic techniques for distinguishing amongst bacterial strains (Vos et al. 1995). Avrova et al. (2002) successfully used AFLPs to study the genetic diversity amongst enterobacteria on potato. In this study, we adapted the AFLP procedure for use with fluorescent dye-labelled primers and an automated DNA sequencer for data capture to reduce the complexity of this technique. As observed previously by Avrova et al. (2002) using AFLPs, fAFLP distinguished all species and subspecies, the taxa clustering into four distinct groups (clusters A–D). These clusters were similar to the clades (A–D) derived by MLST. Avrova et al. (2002) showed that *P. wasabiae* was the most distantly related amongst *Pectobacterium* species using AFLPs. Strains of *P. wasabiae* from New Zealand were also found to

group in a single cluster (Cluster C), and were more distantly related to other species than all remaining *Pectobacterium*. The differences in the relationships between *P. wasabiae* and other *Pectobacterium,* according to AFLP techniques and MLST, may be due to the restricted loci used in multilocus analysis compared to the genomic profile produced by AFLPs. FAFLP further differentiated the isolates of *P. wasabiae*, distinguishing those shown to have identical *acnA* and *mdh* genotypes (e.g. NZEC9 and NZEC8974). Thus, fAFLP fingerprinting is a powerful technique for studying the genetic variation in enterobacteria, providing a simpler alternative to standard AFLP techniques and higher resolution than MLST.

Previous studies investigating the pathogenicity of putative *P. wasabiae* isolates on potato have demonstrated their ability to cause soft rot of tubers as well as stem lesions and wilting (Yap et al. 2004; Glasner et al. 2008; Pitman et al. 2008). Yet, based on PCR and DNA hybridisation results, Kim et al. (2009) found that the putative *P. wasabiae* isolates tested in their study, including strains WPP161, WPP163, WPP168, WPP172, and SCRI 488, lacked a T3SS. Two of the T3SS-deficient strains also lacked genes adjacent to the T3SS gene cluster. The T3SS is important in delivering virulence factors in a variety of Gram-negative pathogens. It has also been shown to be present in strains of *P. atrosepticum* and *P. carotovorum* infecting potato and to be involved in the pathogenicity of phytopathogenic enterobacteria (Rantakari et al. 2001; Holeva et al. 2004; Rojas et al. 2004). PCR and DNA hybridisation were unable to detect the *hrpN* and *dspE* genes in New Zealand isolates belonging to the same clade (Clade C). Given that *hrpN* and *dspE* are indicative of a functional T3SS, their absence suggests that, like similar strains isolated in the USA, *P. wasabiae* from New Zealand possess a divergent T3SS or that the entire cluster encoding the T3SS has undergone excision from the genome. Thus, the T3SS might not be important for virulence in *P. wasabiae*; isolates belonging to this species may use other mechanisms for infection of plant hosts instead.

As another means of investigating the presence of *hrpN* and a functional T3SS in *P. wasabiae*, each strain was tested on tobacco leaves to assess whether any of the isolates could elicit a HR. In contrast to the majority of the strains screened by Kim et al. (2009), we showed that *P. wasabiae* isolates infecting potato in New Zealand can elicit cell collapse. However, several strains were found to produce only a weak response. These results suggest the existence of a functional T3SS in some *P. wasabiae* strains in this study and that they carry a highly divergent T3SS, which cannot be detected by PCR and DNA hybridisation. Certainly, WPP19, tentatively identified as a *P. wasabiae* strain from potato, was shown to hybridise faintly to the T3SS genes only at low stringency and to elicit a weak HR in tobacco plants (Yap et al. 2004).

Three closely related soft rot pathogens of potato *P. atrosepticum* SCRI1043*, P. carotovorum* WPP14 and *P. brasiliensis* 1692 vary in host range and geographical distribution. Recent comparison of their genomes found that each strain possessed a T3SS cluster, including all the genes known to be required to synthesise a functional T3SS (Glasner et al. 2008). Interestingly, only two of the three sequenced strains, *P. carotovorum* WPP14 and *P. brasiliensis* 1692, elicited a type III-dependent HR on infiltration into tobacco leaves, implying that the HR is induced by host recognition of T3SS-secreted effectors. Perhaps the weak HR observed upon infiltration of tobacco leaves with *P. wasabiae* is due to a unique or divergent type III effector.

The *hecB* gene was found to be missing or divergent in *P. wasabiae*, an orthologue of *hecB* being detected only in NZEC12. As the *hecAB* genes are found to flank the *hrp* gene cluster in *P. atrosepticum* and *P. carotovorum* (Bell et al. 2002), this result provides further evidence of rearrangement of the *hrp* cluster and its flanking region in *P. wasabiae*. Furthermore, *hecB* is involved in attachment, aggregation and epidermal cell killing in *Dickeya*, suggesting that *P. wasabiae* strains may be compromised in their ability to attach to the host surface. It would be interesting to compare the epiphytic fitness of *P. wasabiae* and other *Pectobacterium* to determine whether *hecAB* is important for colonisation of potato.

Comparative genomics of *P. wasabiae* strains infecting potato and other hosts would likely help to identify differences in the T3SS in this species of *Pectobacterium*. Genome sequencing might also identify virulence factors other than *hecB* that are responsible for the virulence and host-specificity of these enterobacterial pathogens.

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and *Dickeya paradisiaca* comb. nov. and delineation of four novel species, *Dickeya dadantii* sp. nov., *Dickeya dianthicola* sp. nov., *Dickeya dieffenbachiae* sp. nov. and *Dickeya zeae* sp. nov. *International Journal of Systematic and Evolutionary Microbiology, 55*, 1415–1427.

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