

Soil on imported shipping containers provides a source of new *Pseudomonad* biodiversity into New Zealand

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Abstract Exotic soil attached to shipping containers imported to New Zealand was assessed for new species of *Pseudomonas* (sensu stricto) bacteria to determine whether this soil facilitated the introduction of new biodiversity. Pseudomonads were isolated from exotic soil originating from 19 different countries (termed container isolates (CIs)), and from soils in eight geographically distinct locations throughout New Zealand (termed New Zealand isolates (NZIs)). Partial 16S rRNA gene nucleotide sequences were determined from 40 selected CIs and 38 NZIs. Relationships between CIs, NZIs, and a further 25 typed *Pseudomonad* species were inferred by 16S rRNA phylogenetic analyses. CIs showed relatedness to a broad range of *Pseudomonad* species and many CIs grouped with NZIs suggesting they are closely related to *Pseudomonads* currently in New Zealand. However, unique phylogenetic branchings of CIs also indicate that soils attached to shipping containers may provide a source of new *Pseudomonad* biodiversity to New Zealand.

Keywords *Pseudomonas*; soil; 16S rRNA genes; hypervariable sequence regions; phylogeny

INTRODUCTION

Because New Zealand is an isolated country with consisting of two major islands, the New Zealand Government considers it essential to monitor biological diversity (biodiversity) and prevent the unintentional introduction of potentially invasive species (referred to as “unwanted organisms”). Therefore the New Zealand Biosecurity Act 1993 was established with a major component involving the control of the passage of goods across the border and monitoring these goods for the introduction of “new organisms”. New organisms are defined as: (1) a species of any organism which was not lawfully present in New Zealand on 29 July 1998; (2) a species of any organism which has not been approved for importation for release or release from containment; or (3) an organism which has been eradicated from New Zealand.

Marshall & Varney (2000) first identified soil attached to imported shipping containers (exotic soil) as an unmonitored, potential introductory point for new organisms into New Zealand. This communication reports the focused study on the bacterial genus *Pseudomonas* (sensu stricto) to represent a single “model organism” and access the extent of *Pseudomonad* species diversity entering New Zealand within exotic soils. *Pseudomonads* are considered a taxon of metabolically versatile organisms ubiquitous in soil (Palleroni 1986), play an important role as plant, animal, and human pathogens (Palleroni 1992), and the genus is well characterised and many interspecies relationships documented (Mandel 1966; Palleroni et al. 1972; De Vos & De Ley 1983; De Vos et al. 1985; De Vos et al. 1989; Johnson & Palleroni 1989).

Nucleotide sequence analysis of the 16S rRNA gene was used as the method for comparing *Pseudomonad* species in this study because it is considered an effective method for defining prokaryotic genotypic relatedness and resolving taxonomic identities (Fox et al. 1980; Moore et al. 1996; Head et al. 1998). The 16S rRNA gene from *Pseudomonad* species contains 1492 nucleotide

positions, of which 148 positions are variable and 65 positions of these 148 reside within three hypervariable regions (Moore et al. 1996). These “*Pseudomonas* hypervariable (hv) regions” were defined by Moore et al. (1996) as: hv 1. *E. coli* 16S rRNA gene sequence positions 71–95; hv 2. *E. coli* 16S rRNA gene sequence positions 455–475; and hv 3. *E. coli* 16S gene sequence positions 998–1043— which are located, respectively, within the regions V1: helix 6, V3: helix 18, and V6: helices P35-1 and

P35-2 (Neefs et al. 1990). The “*Pseudomonas* hv 1 region” is recognised as being one of the most variable sequences in 16S rRNAs of bacteria across the phylogenetic spectrum (Woese et al. 1983; Gutell et al. 1985) and is especially useful for reflecting the inferred intragenetic lineages and discerning the type strains of 24 species of the genus *Pseudomonas* (Moore et al. 1996). Furthermore, the cumulative aspect of 16S rRNA sequence databases is particularly important for the analysis of

Table 1 *Pseudomonad* isolates used in this study. (CIs, *Pseudomonads* isolated from exotic soil on imported shipping containers; NZIs, *Pseudomonads* isolated from New Zealand horticultural soil; GenBank, accession numbers of 16S rRNA nucleotide sequences stored in GenBank.)

CIs	Exotic origin	GenBank	NZIs	New Zealand origin	GenBank
C-05E	Australia	AF408942	NZ-LN2	Lincoln	AF408877
C-06A	Australia	AF408941	NZ-LN7	Lincoln	AF408890
C-11E	Northern Europe	AF408904	NZ-LN11	Lincoln	AF408893
C-14B	Singapore	AF408903	NZ-HA1	Hastings	AF408889
C-16C	East Asia	AF408940	NZ-HA3	Hastings	AF408897
C-22B	Pacific Islands	AF408939	NZ-HA6	Hastings	AF408899
C-25B	Pacific Islands	AF408938	NZ-HA7	Hastings	AF408873
C-27B	Pacific Islands	AF408937	NZ-HA8	Hastings	AF408898
C-27C	Pacific Islands	AF408936	NZ-HA9	Hastings	AF408881
C-27D	Pacific Islands	AF408935	NZ-WM2	Waimate	AF408869
C-30E	Japan	AF408934	NZ-WM3	Waimate	AF408868
C-32A	Japan	AF408933	NZ-WM5	Waimate	AF408867
C-34A	Freyberg Wharf	AF408932	NZ-WM7	Waimate	AF408883
C-35B	Export containers	AF408931	NZ-TK3	Takapau	AF408872
C-38C	Pacific Islands	AF408930	NZ-TK7	Takapau	AF408871
C-45D	Australia	AF408929	NZ-TK8	Takapau	AF408870
C-54A	East Coast, United States	AF408928	NZ-TK9	Takapau	AF408869
C-54D	East Coast, United States	AF408927	NZ-WT2	Wellington	AF408891
C-59D	West Coast, United States	AF408926	NZ-WT4	Wellington	AF408864
C-60D	South-west United States	AF408925	NZ-WT6	Wellington	AF408863
C-61E	Northern Europe	AF408924	NZ-WT8	Wellington	AF408879
C-63A	Northern Europe	AF408923	NZ-WT9	Wellington	AF408878
C-66B	Southern Europe	AF408922	NZ-WT10	Wellington	AF408865
C-71C	South Africa	AF408921	NZ-PN3	Palmerston North	AF408886
C-72A	Pacific Islands	AF408920	NZ-PN5	Palmerston North	AF408884
C-74C	South Asia	AF408919	NZ-PN6	Palmerston North	AF408888
C-75D	Japan	AF408918	NZ-PN7	Palmerston North	AF408892
C-80A	East Asia	AF408917	NZ-PN8	Palmerston North	AF408887
C-80E	East Asia	AF408916	NZ-PN9	Palmerston North	AF408894
C-81E	East Asia	AF408915	NZ-CB3	Cambridge	AF408876
C-83A	South-east Asia	AF408914	NZ-CB4	Cambridge	AF408875
C-83D	South-east Asia	AF408913	NZ-CB7	Cambridge	AF408874
C-85A	South-east Asia	AF408902	NZ-PK2	Pukekawa	AF408882
C-85C	South-east Asia	AF408912	NZ-PK4	Pukekawa	AF408880
C-85E	South-east Asia	AF408911	NZ-PK7	Pukekawa	AF408895
C-86A	Singapore	AF408910	NZ-CH2	Christchurch	AF408900
C-86B	Singapore	AF408909	NZ-CH4	Christchurch	AF408896
C-86C	Singapore	AF408908	NZ-CH5	Christchurch	AF408885
C-94A	Export containers	AF408907			
C-96E	Export containers	AF408905			

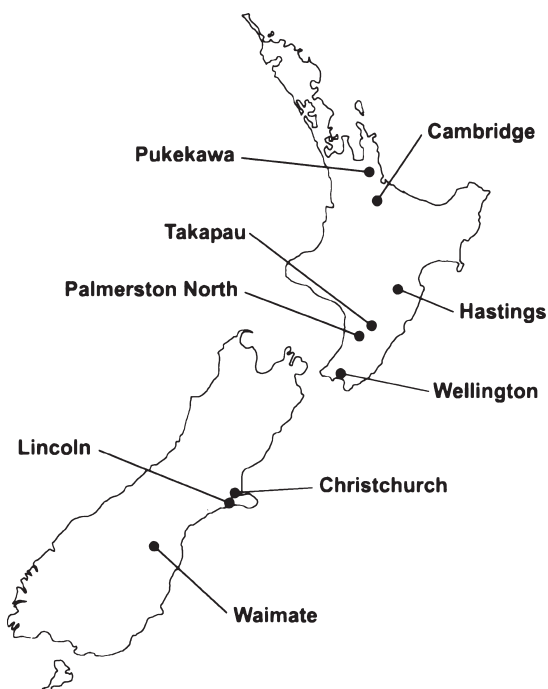


Fig. 1 New Zealand horticultural locations from where soil samples were received in triplicate.

environmental isolates and the recognition of new sources of diversity (Moore et al. 1996). The 16S rRNA (i.e., from a new *Pseudomonad* entering New Zealand) may be added to an existing database and compared with existing and known strains, and therefore, 16S rRNA gene analysis may provide an important tool for rapid monitoring of new bacterial species entering a country.

This communication presents the results of a phylogenetic analysis of nucleotides 1–518 from the 16S rRNA gene (including hv1 and hv2) to address the species diversity between *Pseudomonad* isolates entering New Zealand in exotic soil and *Pseudomonad* isolates currently in New Zealand soils.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Bacterial isolates (Table 1) were maintained at -80°C in Kings B liquid medium (KB) (King et al. 1954) with a final concentration of 20% (v/v) glycerol. Strains were transferred onto *Pseudomonas*

Agar F (PAF) (Difco Laboratories), incubated at 28°C for 16 h and maintained at 4°C for short-term use.

Collection of exotic soil

Cargo ships entering New Zealand with containers originating from a range of international destinations were targeted. Containers were randomly selected (c. 5% of total number entering) to provide exotic soil samples. Soil was collected from surfaces in sterile 500 ml screw-cap specimen containers (BioLab). Soil samples were maintained at 4°C until required.

Collection of New Zealand soil samples

Ten-g soil samples were collected in triplicate within a 300 m^2 area from eight selected horticultural regions throughout New Zealand (Fig. 1). Soil samples were transported at ambient temperature in sealed containers (a period not exceeding 48 h). Upon arrival in the laboratory, soil samples were maintained at 4°C and processed within 72 h.

Selective isolation of *Pseudomonads* from exotic and New Zealand soil samples

One gram of the respective soil sample was placed into a McCartney tube containing sterile KB medium (10 ml). Samples were incubated in an orbital shaker (24 h, 28°C) before serially diluted aliquots (100 μl each) were spread plated onto Gould's agar medium (Gould et al. 1985). Individual bacterial colonies were purified by repeated passage onto fresh Gould's medium and stored as above.

Pseudomonad isolation and selection

Forty *Pseudomonad* isolates were chosen from isolates obtained in a pilot study based on variable Analytical Profile Index (API) 20 NE strip analysis profiles (data presented in Marshall & Varney 2000) and country of origin (Table 1). Exotic soil *Pseudomonad* isolates were termed container isolates (CIs). Thirty-eight *Pseudomonad* isolates (Table 1) from eight New Zealand geographical locations (Fig. 1) were selected based on differences in colony morphology and growth patterns (data not shown). New Zealand soil *Pseudomonads* were termed New Zealand isolates (NZIs).

Genomic DNA isolation and standard PCR conditions

DNA was isolated from pure cultures of bacteria using the Wizard Genomic DNA Isolation Kit (Promega) and stored at 4°C until required. All PCR amplifications were carried out in a Perkin Elmer

9700 thermocycler. Unless stated otherwise, a standard PCR reaction mixture (25 µl total) consisted of 1 × buffer (10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM MgCl₂, 0.01% gelatin, and 0.1% Triton X-100), deoxyribonucleotide triphosphates (dATP, dCTP, dGTP, dTTP) at a final concentration of 200 µM, 0.625 U of *Taq* DNA polymerase (Roche Molecular Biochemicals), various oligonucleotide primers at a final concentration of 2 µM, and 100 ng of template DNA. Thermocycling consisted of 30 cycles (1 min, 94°C; 1 min, 55°C; 1 min, 72°C). Before cycling, samples were heated at 94°C for 5

min and the extension step was increased to 5 min, 72°C as part of the terminal cycle. Primers and dNTPs were removed from PCR products using the High Pure™ PCR Product Purification Kit (Roche Molecular Biochemicals).

PCR amplification and DNA nucleotide sequencing of 16S rRNA gene

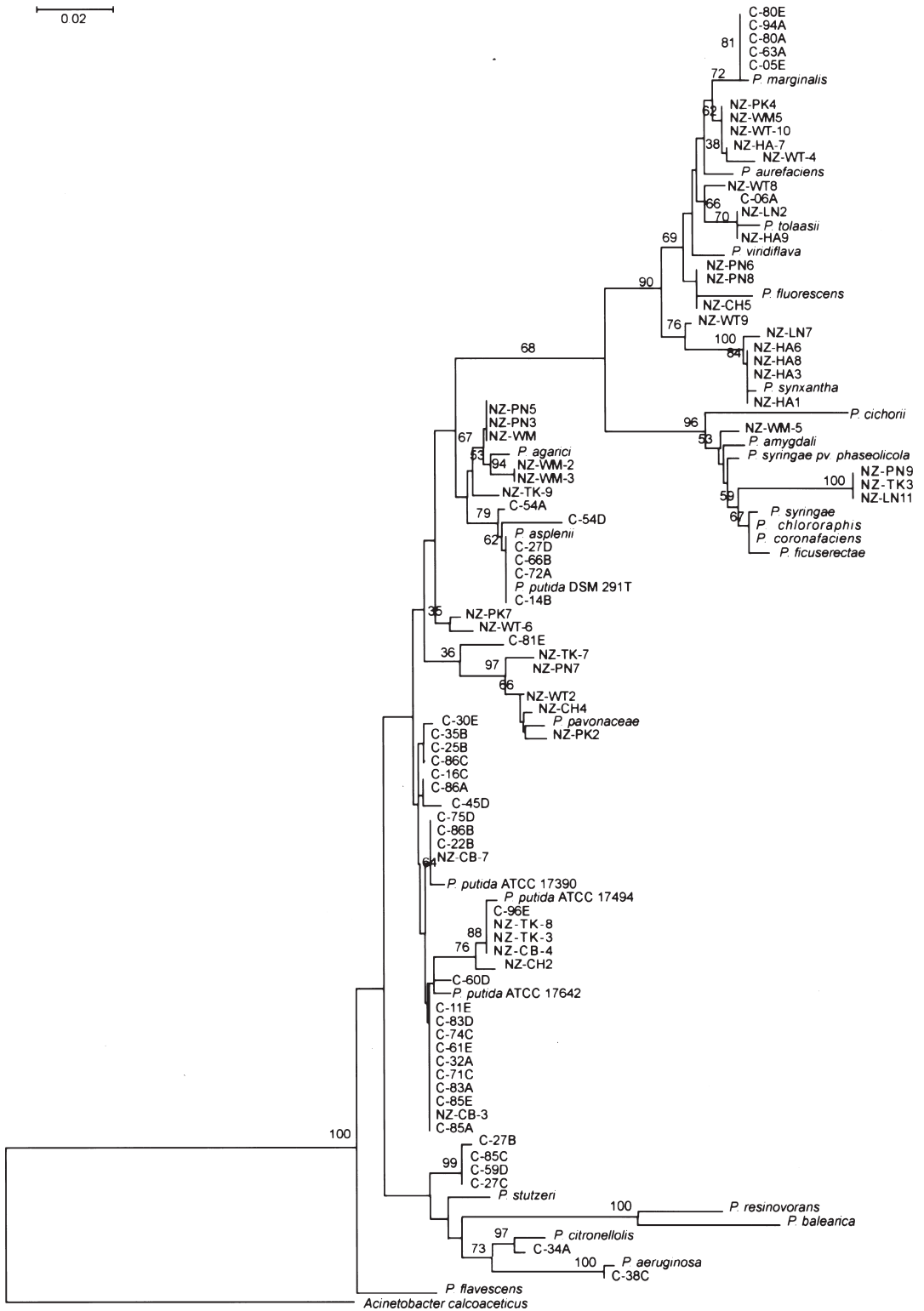
16S rRNA oligonucleotide primers U16A (5'-AGA GTT TGA TCC TGG CTC) and U16B (5'-TAC GGY TAC CTT GTT ACG ACT T) (Wang & Wang 1996) were used to amplify the nearly complete 16S

Table 2 Twenty-five validly described species of the genus *Pseudomonas* (sensu stricto) used in 16S rRNA gene phylogenetic analysis. (ATCC, American Type Culture Collection, Rockville, Maryland, United States; DSM, Deutsche Sammlung von Mikro-organismen, Göttingen, Germany; LMG, Laboratorium voor Microbiologie en Genetica, Rijksuniversiteit, Gent, Belgium; IAM, Institute of Applied Microbiology, Tokyo, Japan; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, United Kingdom.) (T = Type strain.)

Bacterial isolate	Strain designation	GenBank accession
<i>P. aeruginosa</i>	LMG 1242T	Z76651
<i>P. agarici</i>	LMG 2112T	Z76652
<i>P. amygdali</i>	LMG 2123T	Z76654
<i>P. asplenii</i>	LMG 2137T	Z76655
<i>P. aureofaciens</i>	DSM 6698T	Z76656
<i>P. balearica</i>	DAM 6083T	U26418
<i>P. chlororaphis</i>	LMG 5004	Z76657
<i>P. cichorii</i>	LMG 2162T	Z76658
<i>P. citronellolis</i>	DSM 50332T	Z76659
<i>P. coronofaciens</i>	LMG 13190T	Z76660
<i>P. ficuserectae</i>	LMG 5694T	Z76661
<i>P. fluorescens</i> biotype A	DSM 50090T	Z76662
<i>P. marginalis</i> pv. <i>marginalis</i>	LMG 2210T	Z76663
<i>P. pavonaceae</i>	IAM 1155	D84019
<i>P. putida</i>	DSM 291T	Z76667
<i>P. putida</i>	ATCC 17390	AF094741
<i>P. putida</i>	ATCC 17494	AF094740
<i>P. putida</i>	ATCC 17642	AF094741
<i>P. resinovorans</i>	LMG 2274T	Z76668
<i>P. stutzeri</i>	CCUG 11256T	U26262
<i>P. synxanta</i>	DSM 13080	AF267911
<i>P. syringae</i> pv. <i>phaseolicola</i>	MAFF 302282	AB001448
<i>P. syringae</i> (pv. <i>syringae</i>)	LMG 12471T	Z76669
<i>P. tolaasii</i>	LMG 2342T	Z76670
<i>P. viridiflava</i>	LMG 2352T	Z76671
<i>Acinetobacter calcoaceticus</i>	ATCC 23055	Z93434

Fig. 2 Inferred phylogenetic relationships between container isolates and New Zealand isolates from this study and 25 validly described members of the genus *Pseudomonas* (sensu stricto). Culture collection designations are included only for those Pseudomonad species represented by more than one strain. Evolutionary distances were determined with pairwise dissimilarities of the 16S rRNA gene sequences and the dendrogram was generated using the neighbor-joining algorithm. Two major intrageneric clusters and five evolutionary lineages are defined as described by Moore et al. (1996). Bootstrap proportions of confidence are represented as percentages for those branchings with values >50%. ▶

0.02



rRNA gene (c. 1480 bp). Direct nucleotide sequencing of the 16S rRNA nucleotides 1–518 (including hypervariable regions 1 and 2 (hv1 and hv2)) was achieved using primers U16A and R518 (5'-CGT ATT ACC GCG GCT GCT GG) (Lane 1991) in combination with the Big Dye Terminator Kit and an ABI Prism 3TIXLCPE (PE Biosystems). All 16S rRNA gene sequences analysed in this study were confirmed by determining contiguous overlapping sequences of PCR-DNA. The 16S rRNA gene sequences determined in this study have been deposited with GenBank under accession numbers listed in Table 1.

Phylogenetic analysis of 16S rRNA gene nucleotide sequences

The 16S rRNA nucleotide sequences (nucleotides 1–518) obtained in this study were aligned with 25 16S rRNA (nucleotides 1–518) sequences from typed *Pseudomonad* species described in Moore et al. (1996) and selected strains from GenBank (Table 2). Nucleotide alignment was performed using Clustal W software (Thompson et al. 1994). Phylogenetic trees were constructed with neighbor joining (Saitou & Nei 1987) and evolutionary distances calculated according to (Jukes & Cantor 1969) using the software package Treecon for Windows version 1.3b (Van de Peer & De Wachter 1994). Bootstrap analysis (Felsenstein 1985) was carried out using 500 replicates. *Acinetobacter calcoaceticus* ATCC 23055 was included for single sequence (forced) outgroup rooting of the tree.

RESULTS

Phylogenetic characterisation of CIs and NZIs

Figure 2 shows the inferred phylogenetic relationships derived from a neighbor-joining analysis of the pairwise comparisons among the 16S rRNA (nucleotides 1–518) gene sequences of 40 CIs and 38 NZIs from this survey, with 25 selected *Pseudomonad* species (Table 2). All CIs and NZIs were observed to cluster throughout the *Pseudomonas* genus within both the “*P. fluorescens* intrageneric cluster” and the “*P. aeruginosa* intrageneric cluster” (as defined by Moore et al. (1996)).

Of the CIs, six were observed to group closely with *P. marginalis*, one with *P. tolaasii*, five with *P. asplenii*/*P. putida* (note that *P. putida* and *P. asplenii* grouped together as they have been determined (Moore et al. 1996) to have identical 16S

rRNA hv1 nucleotide regions), 22 clustering within a large grouping containing *P. putida* strains (ATCC 17390, 17494, and 17642), four closely related to *P. stutzeri*, one to *P. citronnellolis*, and one to *P. aeruginosa*.

Of the NZIs, five were observed to group together and show close relatedness to *P. marginalis*, three to *P. tolaasii*, three to *P. fluorescens*, six grouped between *P. fluorescens* and *P. cichorii*, one between *P. cichorii* and *P. amygdoli*, three showed close relatedness to *P. syringae* pv. *phaseolicola*, six to *P. agarici*, one related to *P. putida*/*P. asplenii*, two with *P. alcaligenes*, seven with *P. pavonaceae*, and five were observed to group amongst a distinct large branching containing *P. putida* strains (ATCC 17390, 17494, and 17642).

Comparison of CI clusters not containing NZIs, reveals a *P. marginalis* CI cluster, a *P. asplenii*/*P. putida* CI cluster, a *P. putida* ATCC 17514 cluster, a *P. stutzeri* ATCC 17685 CI cluster, an isolate (C-34A) grouping with *P. citronnellolis*, and an isolate (C-38C) grouping with *P. aeruginosa*.

DISCUSSION

Determination of 16S rRNA nucleotide sequences 1–518 from CIs and NZIs in this study included the informative *Pseudomonas* hypervariable regions 1 and 2 (hv1 and hv2). The 16S rRNA hv1 region (in particular, positions 64–103) proved to be highly variable amongst CIs and NZIs, which supports previous studies describing the 16S rRNA hv1 as powerful in the discrimination of *Pseudomonad* species (Woese et al. 1983; Gutell et al. 1985; Moore et al. 1996). The comparison of the 16S rRNA hv2 region (positions 434–473) also showed variability within CIs and NZIs enabling further *Pseudomonad* species discrimination. However, many strains investigated in this study exhibited identical sequence order within both the 16S rRNA hv1 and hv2 regions and these were represented by close phylogenetic grouping. Because identical 16S rRNA hv1 and hv2 regions from isolates originating from the same isolation site (e.g., isolates NZ-HA-8, NZ-HA-3, and NZ-HA-1) were originally selected based on differences in colony morphology and growth patterns, the possibility of these being daughter cells was excluded. Isolates exhibiting identical 16S rRNA hv1 and hv2 were included to represent a quantitative distribution of *Pseudomonad* species isolated in this study and

because there is evidence to show that different Pseudomonad species may still exhibit identical nucleotide order within the 16S rRNA hv1 and hv2 (e.g., *P. aeruginosa* LMG 1242 and *P. stutzeri* CCUG 11256 (Moore et al. 1996)).

Another situation, contrary to the above, has also been demonstrated in which Pseudomonad species having quite distinct 16S rRNA gene profiles, cannot be differentiated phenotypically. For example, *P. stutzeri* is comprised of at least seven genomovars that contain up to six nucleotide differences within the 16S rRNA hv1 region. However, these genomovars could not be differentiated phenotypically (Bennasar et al. 1996).

Analysis of the inferred intragenetic relationships generated in this study (Fig. 2) showed the majority (22 of 42) CIs to group within a large phylogenetic branching most closely related to *P. putida* culture collection strains. *P. putida* presents a taxonomic problem within the genus *Pseudomonas*, in that, although very few differentiating phenotypic characteristics have been determined, the strains of this taxon may be divided into two biotypes, principally on the basis of genomic DNA G+C content (Palleroni 1984). Analysis of the 16S rRNA gene sequences from selected strains classified as *P. putida* has previously shown sequence differences as high as 3% (Moore et al. 1996). As such sequence differences are suggestive of organisms belonging to different species, rather than comprising different strains of the same species, *P. putida* requires further investigation to resolve correct taxonomic assignment.

Further observation of the distribution of CIs within the genus *Pseudomonas*, show many CIs exhibit relatedness to Pseudomonad species associated with pathogenicity. These species include: *P. marginalis* and *P. viridiflava*, which are described as phytopathogens responsible for postharvest rot in cold storage (Lund 1983; Snowdon 1991) and capable of producing enzymes that degrade pectic components in plant cell walls (Collmer & Keen 1986; Liao et al. 1988); *P. tolaasii* which produces a lipodepsipeptide that disrupts cell membranes (Rainey et al. 1993), causing brown blotch of *Agaricus bisporus* (the button mushroom); *P. syringae* is comprised of pathogenic variants (pathovars) that have a wide host range and may cause a variety of disease symptoms on plants (including blights which cause rapid death of tissue, leaf spots and galls) by many enzymatic pathways (Bender et al. 1999); and of medical relevance, *P. aeruginosa* is an opportunistic pathogen responsible for a large number of nosocomial infections and

since its first discovery (Liu 1966) it has been extensively studied (Rocchetta et al. 1999).

When considering the potential risk to New Zealand from CIs that show relatedness with pathogenic Pseudomonad species, one must consider many factors. Although a CI with high nucleotide identity to a known pathogen is suggestive that CI is pathogenic, it would require further assays on numerous hosts to determine true pathogenicity. Many Pseudomonad species are comprised of strains that may be virulent or avirulent, for example, the phytopathogenic species *P. syringae*. *P. syringae* includes more than 40 well characterised pathovars (Palleroni 1984) that cannot be distinguished from one another by standard phenotypic characterisation and require classification by their specificity for a host plant. However, many of these pathovars possess the highly similar 16S rRNA sequences. Therefore, 16S rRNA gene analyses carried out in this study provide an indication of species similarity only and cannot give insight into pathogenic potential. That is, 16S rRNA species-similarity to a known pathogen does not mean that an isolate also contains functional genes that express pathogenicity or, indeed, possesses a phenotype characteristic to that given species. Studies have revealed that horizontal transfer and recombination of virulence genes plays a major role in generating genetic diversity amongst bacterial species (Kehoe et al. 1996) and horizontal gene acquisition has been suggested as a means of why different strains of the species of bacteria have different virulence potentials.

In conclusion: (1) the wide distribution of CIs throughout the *Pseudomonas* genus suggests many diverse Pseudomonad species have the ability to survive in the exotic soil medium during lengthy transportation times; (2) although many phylogenetic branches contain both CIs and NZIs it is likely that some genetic diversity between CIs and NZIs may exist given the above discussions and; (3) phylogenetic branchings that contain exclusively CIs, strongly suggests Pseudomonad species not currently in New Zealand soils. Therefore, exotic soil attached to shipping containers is indeed a likely source of new Pseudomonad biodiversity into New Zealand.

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REFERENCES

- Bender, C. L.; Alarcon-Chaidez, F.; Gross, D. C. 1999: *Pseudomonas syringae* phytotoxins: mode of action, regulation, and biosynthesis by peptide and polyketide synthetases. *Microbiology and Molecular Biology Reviews* 63: 266–292.
- Bennasar, A.; Rossello-Mora, R.; Lalucat, J.; Moore, E. R. B. 1996: 16SrRNA gene sequence analysis relative to genomovars of *Pseudomonas stutzeri* and proposal of *Pseudomonas balearica* sp. nov. *International Journal of Systematic Bacteriology* 46: 200–205.
- Collmer, A.; Keen, N. T. 1986: The role of pectic enzymes in plant pathogenesis. *Annual Review of Phytopathology* 24: 383–409.
- De Vos, P.; De Ley, J. 1983: Intra- and intergeneric similarities of *Pseudomonas* and *Xanthomonas* ribosomal ribonucleic acid cistrons. *International Journal of Systematic Bacteriology* 33: 487–509.
- De Vos, P.; Goor, M.; Gillis, M.; de Ley, J. 1985: Ribosomal ribonucleic acid cistron similarities of phytopathogenic *Pseudomonas* species. *International Journal of Systematic Bacteriology* 35: 169–184.
- De Vos, P.; Landschoot, A. V.; Segers, P.; Tytgat, R.; Gillis, M.; Bauwens, M.; Rossau, R.; Goor, M.; Pot, B.; Kersters, K.; Lizzaraga, P.; De Ley, J. 1989: Genotypic relationships and taxonomic localization of unclassified *Pseudomonas* and *Pseudomonas*-like strains by deoxy-ribonucleic acid:ribosomal ribonucleic acid hybridizations. *International Journal of Systematic Bacteriology* 39: 35–49.
- Felsenstein, J. 1985: Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39: 783–791.
- Fox, G. E.; Stackebrandt, E.; Hespell, R. B.; Gibson, J.; Maniloff, J.; Dyer, T. A.; Wolfe, R. S.; Balch, W. E.; Tanner, R. S.; Magrum, L. J.; Zaben, L. B.; Blakemore, R.; Gupta, R.; Bonen, L.; Lewis, B. J.; Stahl, D. A.; Luehrsen, K. R.; Chen, K. N.; Woese, C. R. 1980: The phylogeny of prokaryotes. *Science* 209: 457–463.
- Gould, W. D.; Hagedorn, C.; Bardinelli, T. R.; Zablutowicz, R. M. 1985: New selective media for enumeration and recovery of fluorescent pseudomonads from various habitats. *Applied and Environmental Microbiology* 49: 28–32.
- Gutell, R. R.; Weiser, B.; Woese, C. R.; Noller, H. F. 1985: Comparative anatomy of 16S-like ribosomal RNA. *Progress in Nucleic Acid Research and Molecular Biology* 32: 155–216.
- Head, I. M.; Saunders, J. R.; Pickup, R. W. 1998: Microbial evolution, diversity, and ecology: a decade of ribosomal RNA analysis of uncultivated microorganisms [Review]. *Microbial Ecology* 35: 1–21.
- Johnson, J. L.; Palleroni, N. J. 1989: Deoxyribonucleic acid similarities among *Pseudomonas* species. *International Journal of Systematic Bacteriology* 39: 230–235.
- Jukes, T. H.; Cantor, C. R. 1969: Evolution of protein molecules. In: Munro, H. N. ed. *Mammalian protein metabolism*. New York, Academic Press. Pp. 21–132.
- Kehoe, M. A.; Kapur, V.; Whatmore, A. M.; Musser, J. M. 1996: Horizontal gene transfer among group A streptococci: implications for pathogenesis and epidemiology. *Trends in Microbiology* 4: 436–443.
- King, E. O.; Ward, M. K.; Raney, D. E. 1954: Two simple media for the demonstration of pyocyanin and fluorescen. *Journal of Laboratory and Clinical Medicine* 44: 301–307.
- Lane, D. J. 1991: 16S/23S sequencing. In: Stackebrandt, E.; Goodfellow, M. ed. *Nucleic acid techniques in bacterial systematics*. Chichester, United Kingdom, John Wiley. Pp. 115–175.
- Liao, C. H.; Hung, H. Y.; Chatterjee, A. K. 1988: An extracellular pectate lysase is the pathogenicity factor of the soft-rotting bacterium *Pseudomonas viridaflava*. *Molecular Plant Microbe Interactions* 1: 199–206.
- Liu, P. V. 1966: The roles of various fractions of *Pseudomonas aeruginosa* in its pathogenesis: identity of the lethal toxins produced in vitro and in vivo. *Journal of Infectious Diseases* 116: 481–489.
- Lund, B. M. 1983: Bacterial spoilage. In: Dennis, C. ed. *Post-harvest pathology of fruits and vegetables*. London, Academic Press. Pp. 219–257.
- Mandel, M. 1966: Deoxyribonucleic acid base composition in the genus *Pseudomonas*. *Journal of General Microbiology* 43: 273–292.

- Marshall, J. W.; Varney, G. 2000: Assessment of contamination soil as a risk pathway. *Report No. 18*, New Zealand Institute for Crop & Food Research Ltd.
- Moore, E. R. B.; Mau, M.; Arnscheidt, A.; Bottger, E. C.; Hutson, R. A.; Collins, M. D.; Van de Peer, Y.; De Wachter, R.; Timmis, K. N. 1996: The determination and comparison of the 16S rRNA gene sequences of species of the genus *Pseudomonas* (sensu stricto) and estimation of the natural intragenetic relationships. *Systematic & Applied Microbiology* 19: 478–492.
- Neefs, J.-M.; Van de Peer, Y.; Hendriks, L.; De Wachter, R. 1990: Compilation of small ribosomal subunit RNA sequences. *Nucleic Acids Research* 18: 2237–2330.
- Palleroni, N. J. 1984: Genus I. *Pseudomonas* Migula 1894. In: Krieg, N. R.; Holt, J. G. ed. *Bergey's manual of systematic bacteriology*. Volume 1. Baltimore, Williams and Wilkins. Pp. 141–198.
- Palleroni, N. J. 1992: Human and animal-pathogenic pseudomonads In: Balows, A.; Truper, H. G.; Dworkin, M.; Harder, W.; Schleifer, K. H. ed. *The prokaryotes, a handbook on the biology of bacteria, ecophysiology, isolation, identification and applications*. Volume 3. New York, Springer. Pp. 3086–3097.
- Palleroni, N. J. 1986: Taxonomy of the Pseudomonads In: Sokatch, J. R. ed. *The bacteria*. London, Academic Press, Inc. Pp. 3–20.
- Palleroni, N. J.; Ballard, R. W.; Ralston, E.; Doudoroff, M. 1972: Deoxyribo-nucleic acid homologies among some *Pseudomonas* species. *Journal of Bacteriology* 110: 1–11.
- Rainey, P. B.; Brodey, C. L.; Johnstone, K. 1993: Identification of a gene cluster encoding three high-molecular-weight proteins, which is required for synthesis of tolaasin by the mushroom pathogen *Pseudomonas tolaasii*. *Molecular Microbiology* 8: 643–652.
- Rocchetta, H. L.; Burrows, L. L.; Lam, J. S. 1999: Genetics of O-antigen biosynthesis in *Pseudomonas aeruginosa*. *Microbiology and Molecular Biology Reviews* 63: 523–553.
- Saitou, N.; Nei, M. 1987: The neighbor joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4: 406–425.
- Snowdon, A. L. 1991: A colour atlas of post-harvest diseases & disorders of fruits and vegetables In: *Vegetables*. Volume 2. Aylesbury, England, Wolfe Scientific Ltd. Pp. 268–271.
- Thompson, J. D.; Higgins, D. G.; Gibson, T. J. 1994: CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22: 4673–4680.
- Van de Peer, Y.; De Wachter, R. 1994: TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. *Computer Applications in the Biosciences* 10: 569–570.
- Wang, G. C.-Y.; Wang, Y. 1996: The frequency of chimeric molecules as a consequence of PCR co-amplification of 16S rRNA genes from different bacterial species. *Microbiology* 142: 1107–1114.
- Woese, C. R.; Gutell, R.; Gupta, R.; Noller, H. E. 1983: Detailed higher-order structure of 16S-like ribosomal ribonucleic acids. *Microbiological Reviews* 47: 621–669.