CHAPTER 6

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Transfer of *Pantoea citrea*, *Pantoea punctata* and *Pantoea terrea* to the genus *Tatumella* emend. as *Tatumella citrea* comb. nov., *Tatumella punctata* comb. nov., and *Tatumella terrea* comb. nov. (Kageyama *et al*., 1992) and the description of *Tatumella morbirosei* sp. nov.

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Summary

Three *Pantoea* species were described for strains isolated from fruit and soil originating in Japan, namely, *Pantoea citrea*, *Pantoea punctata* and *Pantoea terrea*. These three "Japanese" species have been shown to be phylogenetically distant to the remaining species of the genus *Pantoea*. It has previously been observed using multilocus sequence analysis (MLSA) of *Pantoea* strains that the "Japanese" species consistently form a distinct clade with an extended branch length, casting doubt on the inclusion of these species within the genus. Furthermore, the "Japanese" species cluster closely with *Tatumella ptyseos*, strains of which originate from human clinical specimens. DNA-DNA hybridization and phenotypic tests confirmed the phylogenetic distance of *P. citrea*, *P. punctata* and *P. terrea* from the genus *Pantoea* and the affiliation of these species with *Tatumella*. In addition, strains causing pink disease of pineapple, previously identified as *P. citrea*, were proven to be separate species by 16S rRNA, MLSA and DNA-DNA hybridization data. The name *Tatumella morbirosei* sp. nov. (LMG 23360^T = NCPPB 4036^T = CMC6) is proposed for the causal agent of pink disease of pineapple. The new combinations *Tatumella citrea* (Kageyama *et al*. 1992) comb. nov. (LMG 22049^T = ATCC 31623^T = SHS 2003^T), *Tatumella punctata* (Kageyama *et al*. 1992) comb. nov. (LMG 22050^T = ATCC 31626^T = SHS 2006^T) and *Tatumella terrea* (Kageyama *et al*. 1992) comb. nov. (LMG 22051^T = ATCC 31628^T = SHS 2008^T) are proposed for *P. citrea, P. punctata* and *P. terrea*, respectively.

Following the proposal of the novel genus *Pantoea* in 1989 (Gavini *et al*., 1989), but preceding the transfer of *Erwinia ananas* and *Erwinia stewartii* to *Pantoea* (Mergaert *et al*., 1993), three novel *Pantoea* species were described from fruit and soil samples in Japan (Kageyama *et al*., 1992). *Pantoea citrea*, *Pantoea punctata* and *Pantoea terrea* all produced 2,5-diketo-D-gluconic acid (DKGA) from D-glucose and were included in the genus *Pantoea* based on phenotypic data and DNA-DNA hybridization values, despite the inability of other *Pantoea* species to produce DKGA. Until recently, no phylogenetic study had been performed on all validly published *Pantoea* species, giving no reason to doubt the inclusion of *P. citrea*, *P. punctata* and *P. terrea* in the genus *Pantoea*. However, the most recent edition of Bergey's Manual of Systematic Bacteriology (Grimont & Grimont, 2005) states that more taxonomic work is required to justify the assignment of these three species to the genus *Pantoea*.

A recent phylogenetic study of the *Enterobacteriaceae* revealed an *atpD* sequence indel which is specific to *Pantoea* and *Tatumella* (Paradis *et al*., 2005), indicating a close phylogenetic relationship between these two genera. This was in agreement with the initial suggestion of P. Grimont that the "Japanese" species may be more similar to *Tatumella ptyseos* than *Pantoea* in their nutritional patterns (Kageyama *et al*., 1992). The single species genus *Tatumella* was created for clinical strains isolated in North and South America between 1960 and 1980 (Hollis *et al*., 1981). A MLSA scheme based on *rpoB*, *atpD*, *gyrB* and *infB* genes was recently performed on 102 *Pantoea* strains including the "Japanese" species and *Tatumella ptyseos* (Brady *et al*., submitted). A concatenated tree constructed from the sequences of the four genes was found to be the most reliable method for determining phylogenetic relationships amongst *Pantoea* strains. The MLSA study indicated a clear phylogenetic division of *P. citrea*, *P. punctata* and *P. terrea* from the remainder of the *Pantoea* species.

Strains used in this study were obtained from the BCCM/LMG Bacteria Collection (http://www.belspo.be/bccm) and the Centers for Disease Control, Atlanta, Georgia, U.S.A., and are listed in Table 1. An alkalic extraction method was used to isolate genomic DNA from the strains (Niemann *et al*., 1997) which was stored at -20 °C. The complete 16S rRNA gene was amplified and sequenced for the type strains of *P. citrea* (LMG 22049^T), *P. punctata* (LMG 22050^T) and *P. terrea* (LMG 22051^T), as well as one of the additional *P. citrea* strains (LMG 23360) found to cause pink

disease of pineapple (Cha *et al*., 1997) using the primers and conditions determined by Coenye *et al*. (1999). MLSA was performed on all strains (Brady *et al*., submitted).

The GenBank/EMBL accession numbers for the 16S rRNA gene sequences for *Tatumella ptyseos* LMG 7888T, *Tatumella punctata* LMG 22050^T, *Tatumella terrea* LMG 22051^T, *Tatumella citrea* LMG 22049^T and *Tatumella morbirosei* LMG 23360^T are EU344770, EF688006-EF688008 and EU344769, respectively.

The sequences were aligned using ClustalX (Thompson *et al.*, 1997) and the overhangs were trimmed. The Modeltest 3.7 programme (Posada & Crandall, 1998) was then applied to determine the best-fit evolutionary model. Maximum likelihood and neighbour joining analyses were performed using Phyml (Guindon & Gascuel, 2003) and PAUP 4.0b10 (Swofford, 2000) respectively, by applying the models and parameters determined by Modeltest (only Maximum likelihood phylogenetic trees are shown). Bootstrap analysis with 1000 replicates was performed to assess the support for these clusters.

In the 16S rRNA tree (Fig. 1) the genus *Pantoea* is phylogenetically split into two sublineages. The majority of the *Pantoea* "core" species are contained in a cluster supported by high bootstrap support, whilst the "Japanese" species are situated on a distinctly separate branch, also with high bootstrap support of 89 %. These findings support those of Grimont & Grimont, based on 16S rRNA and *rpoB* sequence comparisons, that the "Japanese" species cluster at a lower level to the *Pantoea* "core" clade (2005). Interestingly, the type strain of *Tatumella ptyseos* (LMG 7888^T) clusters closely with the type strain of *Pantoea terrea*, within the "Japanese" species clade. LMG 23360, one of the *P. citrea* strains causing pink disease of pineapple, does not cluster with the type strain of this species (LMG 22049^T), but is found on a separate branch. The 16S rRNA sequence similarity of LMG 23360 was greater than 98 % to the type strains of *P. punctata*, *P. citrea*, *P. septica* and *T. ptyseos*. It is also interesting to note that *P. septica*, *P. eucrina* and *P. dispersa*, which are considered "core" species, cluster separately from the remaining *Pantoea* species in Fig. 1. A similar observation was made in a recent study examining the reliability of 16S rRNA sequence for phylogenetic analysis of the *Enterobacteriaceae* (Naum *et al*., 2008). It

was observed that adding additional animal pathogen sequences to a 16S rRNA phylogenetic analysis would affect the taxon placement of the phytopathogenic enterobacteria, particularly *Erwinia*, *Brenneria* and *Pectobacterium*. This indicated that 16S rRNA gene sequences may be inadequate for determining the true phylogeny of phytopathogenic *Enterobacteriaceae* at the genus level. The discrepancies seen in the 16S rRNA phylogeny can be resolved with the assistance of MLSA.

In Fig. 2, a MSLA phylogenetic tree based on the concatenated sequences of *rpoB*, *atpD*, *gyrB* and *infB* genes, *Pantoea* strains form a monophyletic cluster within the *Enterobacteriaceae*. This cluster contains two sublineages which are supported by high bootstrap values. One of the sublineages consists of the "Japanese" species and the type strain of *T. ptyseos*. The type strain of *T. ptyseos* clusters closely with *P. terrea* strains, specifically with LMG 23565 and its synonym CCUG 30163. This result indicates that *P. terrea* might be a subjective synonym of *T. ptyseos* or another possibility is that only LMG 23565 (syn. CCUG 30163) belongs to *T. ptyseos*. The type strain of *P. citrea* (LMG 22049^T) clusters with the other two *P. citrea* strains causing pink disease of pineapple (LMG 23359 and LMG 23360), but is situated on a separate branch. The distance between the *P. citrea* type strain and the pink disease-causing strains is comparable to the one found between *P. agglomerans* and the newly described *P. eucalypti* (Brady *et al*., submitted), and suggests that LMG 23359 and LMG 23360 do not belong to *P. citrea*.

High quality DNA for DNA-DNA hybridization of strains was prepared by the method of Wilson (1987), with minor modifications (Cleenwerck *et al*., 2002). DNA-DNA hybridizations were performed using the microplate method (Ezaki *et al*., 1989) with some modifications (Cleenwerck *et al*., 2002). The hybridization temperature was 45 °C ± 1 °C and reciprocal reactions were performed with all strains. Representative strains from each "Japanese" *Pantoea* species were selected for hybridization based on the 16S rRNA- and MLSA- phylogenetic trees. The type strains of the "Japanese" species were hybridized amongst each other, and with the type strain of *Tatumella ptyseos*, the phylogenetically closest neighbour, as well as with the type strains of *P. agglomerans*, *P. ananatis*, *P. vagens*, *P. stewartii* ssp. *stewartii* and *P. dispersa*. A summary of the hybridization results is presented in Table 2. The type strains of *P. citrea*, *P. punctata* and *P. terrea* (LMG 22049^T, LMG

22050^T, LMG 22051^T) exhibited 13-21 % DNA similarity when hybridized amongst each other, which is considerably lower than the 28-43 % observed by Kageyama *et al*. (1992) and could be due to different hybridization methods used, namely, the S1 nuclease procedure as described by Johnson (1981). LMG 23360, a strain causing pink disease of pineapple (Cha *et al*., 1997) displayed only 42 % DNA similarity with the type strain of *P. citrea* (LMG 22049^T). This result together with the 16S rRNA and MLSA data proves that LMG 23360 and LMG 23359 do not belong to *P. citrea*. *Pantoea citrea* and *P. punctata* displayed 14 and 21 % DNA relatedness when hybridized to the type strain of *T. ptyseos* (LMG 7888^T), respectively. In contrast, *T. ptyseos* shared 66 % DNA relatedness with the type strain of *P. terrea*, and 87 % DNA relatedness with strain LMG 23565, thought to be *P. terrea*. LMG 23565 demonstrated only 55 % DNA similarity, when hybridized to the type strain of the type strain of *P. terrea*. These results prove that LMG 23565 (syn. CCUG 30163) must be re-classified as *T. ptyseos* and confirm the close phylogenetic relationship between *P. terrea* and *T. ptyseos*, observed in both the 16S rRNA- and MLSA-phylogenetic trees (Figs. 1 & 2).

The DNA relatedness between the type strains of the "Japanese" *Pantoea* species and the type strains of *P. agglomerans*, *P. ananatis*, *P. vagens*, *P. stewartii* and *P. dispersa* was below 10 %. These hybridization values are again lower than those observed by Kageyama *et al*. (1992) between the "Japanese" species and *P. agglomerans* and *P. dispersa* (19 to 22 %). The DNA relatedness values obtained provide further evidence for the exclusion of the "Japanese" species from the genus *Pantoea*.

Based on the close relationship between *T. ptyseos* and *P. terrea*, the consistent clustering of the "Japanese" species with the type strain of *T. ptyseos*, and the DNA hybridization data we propose to transfer *P. citrea*, *P. punctata* and *P. terrea* to the genus *Tatumella* as *Tatumella citrea* comb. nov., *Tatumella punctata* comb. nov. and *Tatumella terrea* comb. nov. We further propose *Tatumella morbirosei* sp. nov. for strains LMG 23359 and LMG 23360, the causal agent of pink disease of pineapple.

The G + C content of the type strains of all four "Japanese" species and *Tatumella ptyseos*, determined by HPLC as published by Mesbah *et al*. (1989), are as follows:

Tatumella citrea comb. nov. (LMG 22049^T) = 49.8 mol %; *Tatumella morbirosei* sp. nov. (LMG 23360^T) = 50.2 mol %; *Tatumella punctata* comb. nov. (LMG 22050^T, LMG 22098) = 50.7 mol %, *Tatumella terrea* comb. nov. (LMG 22051^T, LMG 23564) = 52.6-52.8 mol % and *Tatumella ptyseos* (LMG 7888T, LMG 23565) = 51.7-52.1 mol %.

Physiological and biochemical characteristics for the "Japanese" species were obtained from Bergey's Manual of Systematic Bacteriology (Grimont & Grimont, 2005). Additional tests, including API 50E and Biotype-100 tests (bioMérieux) were performed on the type strains. A summary of distinguishing characteristics of species of the genus *Tatumella* are listed in Table 3. The most important characteristic distinguishing *T. citrea*, *T. morbirosei* sp. nov., *T. punctata, T. terrea* and *T. ptyseos* from *Pantoea* species is their ability to produce 2-ketogluconate dehydrogenase which oxidizes 2-ketogluconate to 2,5-diketo-D-gluconic acid (DKGA). Noticeably, not one *Pantoea* species has this ability but there are other species belonging to the *Enterobacteriaceae* which can produce DKGA from D-glucose, for example, *Pectobacterium cypripedii*, *Ewingella americana*, *Rahnella aquatilis* and *Serratia marcescens* (Bouvet *et al*., 1989).

In a personal communication to Kageyama *et al.*, P. Grimont suggested that the "Japanese" species were phenotypically similar to *Tatumella*, however Kageyama *et al*. (1992) listed several characteristics in which the "Japanese" species differed from *T. ptyseos*. These included acid production from D-xylose and L-arabinose, arginine dihydrolase activity, methyl red reaction, Voges-Proskauer reaction, esculin hydrolase activity and citrate utilization. A closer examination of *T. ptyseos* revealed that this species is in fact positive for the above characteristics listed by Kageyama *et al.* (1992), as are the "Japanese" species. Another reason given by Kageyama *et al.* (1992) not to include the "Japanese" species in the genus *Tatumella* was the G + C content of *T. ptyseos* (Hollis *et al*., 1981), which is 53-54 mol %. They felt that it was too high when compared to the "Japanese" species which range from 49.7 to 51.9 mol %. However, the G + C contents of *P. agglomerans* and *P. dispersa* are 54.9 and 56.9 mol %, respectively which is even higher than that of *T. ptyseos* (Gavini *et al*., 1989).

Emended description of the genus *Tatumella* Hollis, Hickman, Fanning, Farmer, Weaver & Brenner 1981

(Ta.tum.el'la. M.L. dim. Neut. –*ella* ending; M.L. fem. n. *Tatumella* named to honour Harvey Tatum, an American bacteriologist who made many contributions to our understanding of the classification and identification of fermentative and nonfermentative bacteria of medical importance.) The description below is based on the data of Hollis *et al*. (1981), Kageyama *et al*. (1992) and this paper.

Gram-negative, non-capsulated, non-sporeforming small rods that are 0.6-1.2 x 0.9-3.0 µm in size. Cells are motile by means of polar, subpolar or lateral flagella or non-moltile, can be non-motile at 36 °C. Facultatively anaerobic, fermentative, catalyse positive (weak and slow), oxidase negative. Non-pigmented, or pale beige to pale orange. Glucose dehydrogenase, gluconate dehydrogenase and 2-ketogluconate dehydrogenase are produced. Reduce nitrate to nitrite. Indole, urease and gelatin tests are negative. Positive for Voges-Proskauer (Coblentz) and citrate (Simmons), phenylalanine, L-arginine dihydrolase and ONPG tests are variable. Negative for H_2S (TSI), lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, KCN test, lipase and DNase. Acid is produced from D-glucose, D-trehalose, sucrose, D-cellobiose, ribose, L-arabinose, D-arabitol, glycerol, *myo*-inositol, D-mannitol and L-malate, but not from L-sorbose, D-melibiose, D-raffinose, glycogen, histamine, glutarate and propionate. Susceptible to many antibiotics. Isolated from human clinical samples, fruit and soil. The G + C contents range from 49.8 to 53 mol %. The type species is *Tatumella ptyseos* Hollis, Hickman, Fanning, Farmer, Weaver & Brenner 1981.

Description of *Tatumella citrea* (Kageyama, Nakae, Yagi & Sonoyama 1992) comb. nov.

Cells are Gram-negative, short rods (0.8-1.2 x 1.0-3.0 µm) occurring singly or in pairs, non-motile and non-sporeforming. Colonies are pale beige to pale orange, round, convex and smooth with entire margins. Nicotinic acid or nicotinamide are required for growth. Facultatively-anaerobic, oxidase negative, catalase positive, glucose dehydrogenase, gluconate dehydrogenase and 2-ketogluconate dehydrogenase are produced. Indole, urease and phenylalanine deaminase are negative. Reduce nitrate to nitrite. The following carbon sources are utilized at 28 °C within three to six days: trans-aconitate, D-adonitol (weak), L-arabinose, D-arabitol, L-arabitol (weak), D-cellobiose (weak), citrate, erythritol, D-fructose, D-galactose, D-galacturonate, D-glucose, inositol, 5-ketogluconate, lactose (weak), lactulose, D-malate, D-maltose, maltotriose, D-mannitol, quinate, L-rhamnose, D-ribose, sucrose, D-tagatose, L-tartrate, meso-tartrate, D-trehalose, xylitol (weak) and D-xylose. The following carbon sources are not utilized at 28 °C within three to six days: L-alanine, betain, dulcitol, L-fucose, gentiobiose, glutarate, histamine, D-mannose, D-melibiose, propionate, D-raffinose, D-sorbitol, L-sorbose, D-tartrate, trigonelline, L-tryptophan, D-turanose, L-tyrosine.

The G + C content of the type strain is 49.8 mol %. The type strain is LMG 22049^T (= ATCC 31623^T = SHS 2003^T) and was isolated from mandarin orange in Japan.

Description of *Tatumella punctata* (Kageyama, Nakae, Yagi & Sonoyama 1992) comb. nov.

Cells are Gram-negative, short rods (1.1-1.2 x 1.3-2.3 µm) occurring singly or in pairs, non-motile and non-sporeforming. Colonies are pale beige to pale orange, round, convex and smooth with entire margins. Nicotinic acid or nicotinamide are required for growth. Facultatively-anaerobic, oxidase negative, catalase positive, glucose dehydrogenase, gluconate dehydrogenase and 2-ketogluconate dehydrogenase are produced. Indole, urease and phenylalanine deaminase are negative. Reduce nitrate to nitrite. The following carbon sources are utilized at 28 °C within three to six days: trans-aconitate, L-alanine, L-arabinose, D-arabitol, D-cellobiose, citrate, dulcitol (weak), erythritol, D-fructose, D-galactose, D-galacturonate, gentiobiose, D-glucose, inositol, 5-ketogluconate, lactulose (weak), D-malate (weak), D-maltose, maltotriose, D-mannitol, D-mannose, quinate (weak), L-rhamnose, D-ribose, D-sorbitol (weak), sucrose, D-tagatose (weak), L-tartrate, meso-tartrate, D-trehalose, D-turanose (weak) and D-xylose. The following carbon sources are not utilized at 28 °C within three to six days: D-adonitol, L-arabitol, betain, L-fucose, glutarate, histamine, lactose, D-melibiose, propionate, D-raffinose, L-sorbose, D-tartrate, trigonelline, L-tryptophan, L-tyrosine and xylitol.

The G + C content of the type strain is 50.7 mol %. The type strain is LMG 22050^T (= ATCC 31626^T = SHS 2006^T) and was isolated from mandarin orange in Japan.

Description of *Tatumella terrea* (Kageyama, Nakae, Yagi & Sonoyama 1992) comb. nov.

Cells are Gram-negative, short rods (0.8-0.9 x 1.2-2.0 µm) occurring singly or in pairs, motile by means of one or two lateral flagella and non-sporeforming. Colonies are pale beige to pale orange, round, convex and smooth with entire margins. Nicotinic acid or nicotinamide are required for growth. Facultatively-anaerobic, oxidase negative, catalase positive, glucose dehydrogenase, gluconate dehydrogenase and 2-ketogluconate dehydrogenase are produced. Indole, urease and phenylalanine deaminase are negative. Reduce nitrate to nitrite. The following carbon sources are utilized at 28 °C within three to six days: trans-aconitate (weak), D-adonitol (weak), L-arabinose, D-arabitol, D-cellobiose, citrate, erythritol (weak), D-fructose, D-galactose, D-galacturonate (weak), gentiobiose, D-glucose, inositol, 5-ketogluconate, lactulose, D-malate, D-maltose, maltotriose, D-mannitol, D-mannose, quinate (weak), L-rhamnose, D-ribose, sucrose, D-tagatose, meso-tartrate (weak), D-trehalose and D-xylose. The following carbon sources are not utilized at 28 °C within three to six days: L-alanine, L-arabitol, betain, dulcitol, L-fucose, glutarate, histamine, lactose, D-melibiose, propionate, D-raffinose, D-sorbitol, L-sorbose, D-tartrate, L-tartrate, trigonelline, L-tryptophan, D-turanose, L-tyrosine, xylitol.

The G + C content of the type strain is 52.8 mol %. The type strain is LMG 22051^T = ATCC 31628^T = SHS 2008^T) and was isolated from soil in Japan.

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Description of *Tatumella morbirosei* sp. nov.

Tatumella morbirosei (mor.bi.ró.se.i. L. n. *morbus* meaning disease and L. adj. *roseus* meaning rosy, pink. N.L. gen. N. *morbirosei*, of the pink disease, referring to the causal agent of pink disease of pineapple)

Cells are Gram-negative, short rods (0.8-1.2 x 1.0-3.0 µm) occurring singly or in pairs, non-motile and non-sporeforming. Colonies are pale beige, round, convex and smooth with entire margins. Facultatively-anaerobic, oxidase negative, catalase positive, glucose dehydrogenase, gluconate dehydrogenase and 2-ketogluconate dehydrogenase are produced. Indole and urease are negative, phenylalanine deaminase is weakly positive. Reduce nitrate to nitrite. The following carbon sources are utilized at 28 °C within three to six days: trans-aconitate, D-adonitol, L-arabinose, D-arabitol, D-cellobiose, citrate, erythritol, D-fructose, D-galactose, D-galacturonate, D-glucose, inositol, 5-ketogluconate, lactulose, D-malate, D-maltose, maltotriose, D-mannitol, D-mannose, quinate (weak), L-rhamnose, D-ribose, sucrose, D-tagatose, meso-tartrate (weak), D-trehalose, trigonelline, L-tyrosine and D-xylose. The following carbon sources are not utilized at 28 °C within three to six days: L-alanine, L-arabitol, betain, dulcitol, L-fucose, gentiobiose, glutarate, histamine, lactose, D-melibiose, propionate, D-raffinose, D-sorbitol, L-sorbose, D-tartrate, L-tartrate, L-tryptophan, D-turanose and xylitol.

The G + C content of the type strain is 50.2 mol %. The type strain is LMG 23360^T (= NCPPB 4036 = CMC6) and was isolated from pineapple in the Philippines.

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Table 1: Strains of *Pantoea* and *Tatumella* used in this study, LMG = BCCM/LMG Bacteria Collection, Ghent University, Belgium, ATCC = American Type Culture Collection, Rockville, Maryland, U.S.A., CCUG = Culture Collection, University of Göteborg, Sweden, CDC = Centres for Disease Control, Atlanta, Georgia, U.S.A.

^{T} = type strain

Species	Strain	Host	Location
Pantoea agglomerans	LMG 1286^{T}	Human	Zimbabwe
	LMG 2660	*Wisteria floribunda*	Japan
Pantoea ananatis	LMG 2665^{T}	Pineapple	Brazil
	LMG 20103	*Eucalyptus*	South Africa
Pantoea stewartii ssp. *stewartii*	LMG 2715^{T}	Maize	USA
	LMG 2718	Maize	USA
Pantoea stewartii ssp. *indologenes*	LMG 2632^{T}	Fox millet	India
	LMG 2673	Pineapple	Hawaii
Pantoea dispersa	LMG 2603^{T}	Soil	Japan
	LMG 2604	Wild rose	Netherlands
Pantoea anthophila	LMG 2558^{T}	*Impatiens balsamina*	India
	LMG 2560	*Tagetes erecta*	Unknown
Pantoea vagens	LMG 24199^{T}	*Eucalyptus*	Uganda
	LMG 24201	Maize	South Africa
Pantoea eucalypti	LMG 24198^{T}	*Eucalyptus*	Uruguay
	LMG 24197	*Eucalyptus*	Uruguay
Pantoea deleyii	LMG 24200^{T}	*Eucalyptus*	Uganda
Pantoea septica	LMG 5345^{T}	Human, stool	New Jersey, USA
	LMG 24526	Human, blood	New York, USA
Pantoea eucrina	LMG 2781^{T}	Human, trachea	Connecticut, USA
	LMG 24529	Human, cyst	Georgia, USA

Pantoea brenneri	LMG 5343^{T}	Human, urethra	Montana, USA
	LMG 24532	Human, sputum	Wisconsin, USA
Pantoea conspicua	LMG 24534^{T}	Human, blood	Paris, France
Tatumella citrea	LMG 22049^{T} = SHS 2003	Mandarin orange	Japan
Tatumella morbirosei	LMG 23360^{T} = NCPPB 4036^{T}	Pineapple	Philippines
	LMG 23359 = NCPPB 4035	Pineapple	Philippines
Tatumella punctata	LMG 22050^{T} = SHS 2006	Mandarin orange	Japan
	LMG 22097 = SHS 2004	Mandarin orange	Japan
	LMG 22098 = SHS 2005	Persimmon	Japan
	LMG 23562 = SHS 2004	Mandarin orange	Japan
	LMG 23563 = SHS 2007	Mandarin orange	Japan
	CCUG 30157 = SHS 2004	Mandarin orange	Japan
	CCUG 30160 = SHS 2007	Mandarin orange	Japan
Tatumella terrea	LMG 22051^{T} = SHS 2008	Soil	Japan
	LMG 23564 = SHS 2009	Soil	Japan
	CCUG 30162 = SHS 2009	Soil	Japan
Tatumella ptyseos	LMG 7888^{T} = ATCC 33301^{T}	Human	USA
	LMG 23565 = SHS 2010	Soil	Japan
	CCUG 30163 = SHS 2010	Soil	Japan

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Table 2: DNA-DNA hybridization values between *T. citrea* comb. nov. (LMG 22049^T) *T. morbirosei* sp. nov. (LMG 23360), *T. punctata* comb. nov. (LMG 22050^T, LMG 22098), *T. terrea* comb. nov. (LMG 22051^T, LMG 23564), *T. ptyseos* (LMG 7888^T, LMG 23565), *P. agglomerans* (LMG 1286^T), *P. ananatis* (LMG 2665^T), *P. vagens* (LMG 24199^T), *P. stewartii* ssp. *stewartii* (LMG 2715^T) and *P. dispersa* (LMG 2603^T).

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
1. LMG 22049^T	100												
2. LMG 23360	42	100											
3. LMG 22050^T	21	17	100										
4. LMG 22098	16		93	100									
5. LMG 22051^T	13	11	17	18	100								
6. LMG 23564					82	100							
7. LMG 23565	15	13	20		55	54	100						
8. LMG 7888^T	14	15	21		66		87	100					
9. LMG 1286^T	6		8		8				100				
10. LMG 2665^T	5		6		6				21	100			
11. LMG 24199^T	5		7		8				65	20	100		
12. LMG 2715^T	2		3		3				6	20	9	100	
13. LMG 2603^T	7		7	7	8				24	20	19	22	100

Table 3: Phenotypic characteristics distinguishing *T.citrea* comb. nov., *T. morbirosei* sp. nov., *T. punctata* comb. nov. and *T.terrea* comb. nov. from each other and from *T. ptyseos*

1 = *T. citrea*, 2 = *T. morbirosei*, 3 = *T. punctata*, 4 = *T. terrea*, 5 = *T. ptyseos*

+, 90-100 % of strains positive in 1-2 days; (+), 90-100 % of strains positive in 1-4 days; -, 90-100 % of strains negative in 4 days

Characteristic	**1**	**2**	**3**	**4**	**5**
Phenylalanine deaminase	-	+	-	-	+
Arginine dihydrolase	+	+	+	-	(+)
Lactose	+	-	-	-	+
Gentiobiose	-	-	+	+	(+)
D-Xylose	+	-	+	+	+
L-Rhamnose	+	-	+	+	+
L-Tartrate	+	-	+	-	+
Trigonelline	-	+	-	-	-
D-Arabinose	-	+	+	-	-

Figure 1: Maximum likelihood tree based on complete 16S rRNA sequences of *Tatumella* and *Pantoea* species. The tree was generated by the Phyml software using the Tamura-Nei (TN93) model as selected by Modeltest. Bootstrap values after 1000 replicates are expressed as percentages. *Escherichia coli* was included as an outgroup.

NCTC 9381^{T}|*Pantoea agglomerans*|AJ25166

LMG 24199^{T}|*Pantoea vagens*|EF688012

LMG 24198^{T}|*Pantoea eucalypti*|EF688009

LMG 2558^{T}|*Pantoea anthophila*|EF688010

54

LMG 24534^{T}|*Pantoea conspicua*|EU216737

LMG 24200^{T}|*Pantoea deleyii*|EF688011

99

LMG 5343^{T}|*Pantoea brenneri*|EU216735

82

LMG 2665^{T}|*Pantoea ananatis*|Z96081

LMG 2715^{T}|*Pantoea stewartii* ssp. *stewartii* |Z96080

100

LMG 2632^{T}|*Pantoea stewartii* ssp. *indologenes*|Y13251

LMG 7888^{T}|*Tatumella ptyseos*|AJ233437

99

53

LMG 22051^{T}|*Tatumella terrea*|EF688007

91

41

LMG 22050^{T}|*Tatumella punctata*|EF688006

89

LMG 23360^{T}|*Tatumella morbirosei*|EU344769

LMG 22049^{T}|*Tatumella citrea*|EF688008

81

LMG 7871^{T}|*Kluyvera ascorbata*|AF008579

27

LMG 7870^{T}|*Raoultella planticola*|AB004756

70

64

DSM 30039^{T}|*Citrobacter freundii*|AJ233408

ATCC 49162^{T}|*Enterobacter hormaechei*|AJ508302

60

LMG 5345^{T}|*Pantoea septica*|EU216734

LMG 2781^{T}|*Pantoea eucrina*|EU216736

93

LMG 2603^{T}|*Pantoea dispersa*|DQ504305

LMG 2024^{T}|*Erwinia amylovora*|Z96088

98

67

DSM 12162|*Erwinia pyrifoliae*|AJ009930

LMG 2613^{T}|*Erwinia billingiae*|Y13249

ATCC 11775^{T}|*Escherichia coli*|X80725

0.01

Figure 2: Maximum likelihood tree based on concatenated partial sequences of *rpoB*, *atpD*, *gyrB* and *infB* of *Tatumella* and *Pantoea* strains. The tree was generated by the Phyml software using the general time reversible (GTR) model as selected by Modeltest. Bootstrap values after 1000 replicates are expressed as percentages. *Shigella dysenteriae* sequences were obtained from the genome sequencing database of the Sanger Institute (http://www.sanger.ac.uk) and included as an outgroup.

100 LMG 2660|*Pantoea agglomerans*
99 LMG 1286^{T}|*Pantoea agglomerans*
100 LMG 24198^{T}|*Pantoea eucalypti*
61 LMG 24197|*Pantoea eucalypti*
100 LMG 24201|*Pantoea vagens*
44 LMG 24199^{T}|*Pantoea vagens*
100 LMG 2560|*Pantoea anthophila*
LMG 2558^{T}|*Pantoea anthophila*
100 LMG 24200^{T}|*Pantoea deleyii*
100 LMG 5343^{T}|*Pantoea brenneri*
99 LMG 24532|*Pantoea brenneri*
LMG 24534^{T}|*Pantoea conspicua*
97
LMG 2718|*Pantoea stewartii* ssp. *stewartii*
LMG 2715^{T}|*Pantoea stewartii* ssp. *stewartii*
100 LMG 2673|*Pantoea stewartii* ssp. *indologenes*
50
100 LMG 2632^{T}|*Pantoea stewartii* ssp. *indologenes*
100 LMG 2665^{T}|*Pantoea ananatis*
LMG 20103|*Pantoea ananatis*
100
100 LMG 5345^{T}|*Pantoea septica*
LMG 24526|*Pantoea septica*
100 LMG 2781^{T}|*Pantoea eucrina*
99 LMG 24529|*Pantoea eucrina*
100 LMG 2604|*Pantoea dispersa*
LMG 2603^{T}|*Pantoea dispersa*
LMG 23562|*Tatumella punctata*
CCUG30157|*Tatumella punctata*
LMG22097|*Tatumella punctata*
100 LMG 22050^{T}|*Tatumella punctata*
LMG23563|*Tatumella punctata*
LMG22098|*Tatumella punctata*
99 CCUG30160|*Tatumella punctata*
100 LMG 23359|*Tatumella morbirosei*
100 LMG 23360^{T}|*Tatumella morbirosei*
100 LMG 22049^{T}|*Tatumella citrea*
LMG23565|*Tatumella ptyseos*
100 CCUG30163|*Tatumella ptyseos*
LMG 7888^{T}|*Tatumella ptyseos*
100 LMG 23564|*Tatumella terrea*
CCUG30162|*Tatumella terrea*
100 LMG 22051^{T}|*Tatumella terrea*
Shigella dysenteriae

0.1

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CHAPTER 7

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Isolation of *Enterobacter cowanii* from *Eucalyptus* showing symptoms of bacterial blight and dieback in Uruguay

As prepared for: Letters in Applied Microbiology

ABSTRACT

Aims: This study was performed to identify bacterial strains isolated simultaneously with *Pantoea* species from *Eucalyptus* trees showing symptoms of bacterial blight and dieback in Uruguay.

Methods and Results: Several molecular techniques including 16S rRNA- and *rpoB*-gene sequencing and DNA-DNA hybridization were used to characterize the Gram-negative, facultatively-anaerobic, slime-producing bacterial strains isolated along with *Pantoea* species from *Eucalyptus*. Hypersensitivity reactions and pathogenicity tests were performed on tobacco and *Eucalyptus* seedlings, respectively. The isolates clustered closely with the type strain of *E. cowanii* in both phylogenetic trees constructed. The DNA-DNA similarity between the isolates and the type strain of *E. cowanii* ranged from 88-92 %. A positive hypersensitivity reaction was observed on the tobacco seedlings, but no disease symptoms were visible on the inoculated *Eucalyptus* seedlings.

Conclusions: *E. cowanii* was isolated from trees with symptoms of bacterial blight, although strains of this bacterial species do not appear to be the causal agent of the disease.

Significance and Impact of Study: This study provides the first report of *E. cowanii* isolated from *Eucalyptus*. Its presence in *Eucalyptus* tissue suggests that it is an endophyte in trees showing symptoms of blight.

INTRODUCTION

Eucalyptus grandis trees in Uruguay commonly exhibit symptoms of leaf blight and dieback disease. The cause of this disease is unknown, but symptoms such as water-soaked lesions with a greasy appearance are typical of bacterial infections. In 2002, symptomatic leaves and shoots were collected and isolations were made from the infected tissue. Gram-negative, facultatively-anaerobic bacteria were consistently isolated from the diseased material. The majority of the strains were yellow-pigmented and were thought to belong to *P. ananatis*, the causal agent of bacterial blight and dieback on *Eucalyptus* in South Africa (Coutinho *et al*., 2002). Several non-pigmented, slime-producing strains were also isolated from the diseased material. The yellow-pigmented strains were subsequently identified as representing three novel species belonging to the genus *Pantoea* using multilocus sequence analysis (MLSA) based on *rpoB*, *atpD*, *gyrB* and *infB* gene sequences as a supporting technique (Brady *et al*., submitted). The aim of this study was to identify the non-pigmented, slime-producing bacterial strains isolated together with *Pantoea* species from *E. grandi*s leaves and shoots in Uruguay. The strains were identified using 16S rRNA- and *rpoB* gene-sequence comparisons as well as DNA-DNA hybridization. In addition pathogenicity tests were performed on *Eucalyptus* seedlings to consider their possible role in causing disease, as it was not clear whether the *Pantoea* strains or the non-pigmented strains were responsible for the leaf- and shoot-blight symptoms observed.

MATERIALS AND METHODS

Bacterial strains and DNA extraction

Five non-pigmented slime-producing strains were isolated from *Eucalyptus* leaves showing typical bacterial blight symptoms including leaf spots and water-soaked lesions. The leaves were surface-disinfected, crushed in sterile water and the resulting suspension was streaked on nutrient agar and incubated at 28 °C for three days. Single colonies were obtained by re-streaking and incubation under the same conditions. Genomic DNA was extracted from each of the bacterial strains using an alkalic extraction method (Niemann *et al*., 1997) and stored at -20 °C. Strains used in this study are listed in Table 1 and are maintained at the Forestry and Agricultural Biotechnology Institute (FABI).

16S rRNA and *rpoB* gene sequencing and analysis

The complete 16S rRNA sequence was determined for two representative strains using the primers and conditions determined by Coenye *et al.* (1999). The representative strains were selected from clusters (data not shown) generated by an AFLP technique developed for the genus *Pantoea* (Brady *et al*., 2007). *rpoB* gene sequencing was performed on all five strains using the method described in the MLSA scheme developed for the genus *Pantoea* (Brady *et al*., submitted). The sequences were aligned using ClustalX (Thompson *et al*., 1997) and the overhangs trimmed. The Modeltest 3.7 programme (Posada and Crandall, 1998) was then applied to the data sets to determine the best-fit evolutionary model to apply to each gene. Maximum likelihood analysis was performed using Phyml (Guindon and Gascuel, 2003), by applying the models and parameters determined by Modeltest. Bootstrap analysis with 1000 replicates was performed on the trees to assess the reliability of the clusters.

DNA-DNA hybridization and G + C content

High quality DNA for DNA-DNA hybridization of strains was prepared using the method of Wilson (1987), with minor modifications (Cleenwerck *et al*., 2002). DNA-DNA hybridizations were performed using the microplate method (Ezaki *et al*., 1989) with some modifications (Cleenwerck *et al*., 2002). The hybridization temperature

was 45 °C ± 1 °C and reciprocal reactions were performed with DNA from all strains. The type strain of *Enterobacter cowanii* (LMG 23569^{T}) was hybridized to BCC 009 and BCC 078, and BCC 009, BCC 011 and BCC 078 were hybridized amongst each other. The G + C contents of the strains were determined by HPLC as published by Mesbah *et al.* (1989).

Pathogenicity tests

Hypersensitivity reaction (HR) tests were conducted on four tobacco seedlings (*Nicotiana tabacum*) by injecting a bacterial suspension of 10^{8} CFU/ml of strains BCC 008, BCC 011, BCC 074 and BCC 078 into the intercellular spaces of the leaves with a fine needle and syringe. Pathogenicity tests were performed on 12 cuttings of a susceptible *E. grandis* x *E. nitens* clone as previously described (Coutinho *et al.*, 2002). Seedling leaves were inoculated with sterile water as a negative control and with LMG 20103 (*Pantoea ananatis* pathogenic on *Eucalyptus*) as a positive control. The seedlings were covered with plastic bags to induce humid conditions and incubated for two weeks. Cutting were assessed by using a 0 to 3 scale (0 = no disease, 3 = lesion larger than 1 cm).

RESULTS

Sequence analyses

The GenBank/EMBL accession numbers for the 16S rRNA gene sequences for BCC 009 and BCC 078 are EU629163 and EU629164, respectively; and EU629165-EU629169 for the *rpoB* genes for strains BCC 008, BCC 009, BCC 011, BCC 074 and BCC 078. The 16S rRNA sequences of strains BCC 009 and BCC 078 was greater than 99.8 % similar to *E. cowanii* and greater than 98 % similar to *E. cloacae*, *E. radicincitans*, *E. asburiae* and *E. cancerogenus*. In the 16S rRNA phylogenetic tree (Fig. 1), BCC 009 and BCC 078 clustered with the type strain of *E. cowanii* with a strong bootstrap support of 79 %. All five strains clustered closely with the type strain of *E. cowanii* in the *rpoB* phylogenetic tree with high bootstrap support of 100 % (Fig. 2). The topologies of the 16S rRNA- and *rpoB*- trees were similar to those of Stephan *et al.* (2007).

DNA-DNA hybridization and G + C content

When hybridized to BCC 009 and BCC 078, the type strain of *E. cowanii* (LMG 23569^T) exhibited 92 % and 88 % DNA similarity, respectively. The DNA similarity amongst strains BCC 009, BCC 011 and BCC 078 ranged from 76 to 92 %. The G + C contents for strains BCC 008, BCC 009, BCC 011, BCC 074 and BCC 078 ranged from 55.8 to 56.6 mol %, which is similar to the 53 mol % of the type strain of *E. cowanii*, LMG 23569^T published by Grimont and Grimont (2005a).

Pathogenicity tests

A positive hypersensitivity reaction was observed on the tobacco seedlings inoculated with strains BCC 008, BCC 011, BCC 074 and BCC 078, which was demonstrated by the complete collapse of the leaf tissue after 24 hours. The inoculated *Eucalyptus* seedlings and the negative water control displayed no symptoms during the two weeks in which the leaves were examined (score obtained = 0). In contrast, the leaves inoculated with *P. ananatis* developed necrotic lesions within five days of inoculation (score obtained = 3).

DISCUSSION

Results of this study demonstrated that the non-pigmented, slime-producing bacterial strains isolated from the internal parts of *Eucalyptus* tissue together with *Pantoea* species, are *E. cowanii*. This identification was clear from the phylogenetic trees based on 16S rRNA- and *rpoB*-gene sequence comparisons, both of which were strongly supported by high bootstraps.The sequencing results were confirmed by DNA-DNA hybridization data and G + C content of the strains. The DNA-DNA similarity values of BCC 009 and BCC 078 with LMG 23569^T, the type strain of *E. cowanii* and between strains BCC 009, BCC 011 and BCC 078, are both well above the recommended species definition cut-off of 70 % (Wayne *et al.*, 1987). Additionally, the G + C contents of the strains are within the 5 mol % difference range for species delineation (Rosselló-Mora and Amann, 2001).

Species belonging to the genus *Enterobacter* are typically associated with the environment. Some cause diseases of trees, while others are opportunistic human pathogens (Grimont and Grimont, 2006). The occurrence of *E. cowanii* on *Eucalyptus* in this study was interesting as the bacterium has never previously been isolated from this tree, where it evidently can live internally in leaf tissue. *Enterobacter cowanii* was described for a group of clinical strains which were previously identified as *E. agglomerans* (*Pantoea agglomerans*) in routine diagnostic laboratories (Inoue *et al*., 2000). Of the 15 recognized species belonging to *Enterobacter*, only three have previously been isolated from diseased trees, *E. cancerogenus*, *E. nimipressuralis* and *E. pyrinus* (Grimont and Grimont, 2005a). In this regard, the occurrence of the bacterium on *Eucalyptus* is perhaps not unusual.

The pathogenicity tests indicated that *E. cowanii* is unlikely to have played a part in the bacterial blight and dieback of *Eucalyptus* in Uruguay, although the hypersensitivity reactions on tobacco seedlings were positive. It is possible that *E. cowanii* can contribute to disease under certain environmental conditions favourable to the bacterium. However, it is likely that *E. cowanii* isolated in this study is an endophyte that was coincidentally isolated together with *Pantoea* species which may cause the leaf symptoms observed.

The view that *Enterobacter cowanii* isolated in this study is most likely an endophyte of the *Eucalyptus* tissue from which it was isolated in this study, is consistent with the ecology of other *Enterobacteriaceae*. For example, *E. cloacae* has been found as an endophytic symbiont of corn (Hinton and Bacon, 1995) and papaya (Pious *et al*., 2007) and as an obligatory endophyte of Mediterranean pines (Madmony *et al*., 2005). *E. asburiae* is a well known endophyte (Quadt-Hallmann *et al*., 1997) and *E. gergoviae* is an opportunistic endophyte of maize (An *et al*., 2007). These three *Enterobacter* species are known for their regular isolation from clinical samples, in addition to *E. cloacae* causing nosocomial infections (Grimont and Grimont, 2006). There are other examples within the family *Enterobacteriaceae* of species causing human disease or being isolated from clinical samples but also existing as phytopathogens or endophytes, especially for species residing in the genus *Pantoea*. *P. agglomerans* is considered a rare opportunistic pathogen but also causes disease on plant hosts and *P. ananatis* causes a range of plant and agricultural diseases but has

also been isolated from septic patients (Grimont and Grimont, 2005b). This emphasizes the ubiquitous nature of both *Enterobacter* and *Pantoea* species.

This study represents the first report of *E. cowanii* isolated from diseased *Eucalyptus*. The bacterium does not appear to be involved in the bacterial blight and dieback in Uruguay. Nonetheless, its occurrence in *Eucalyptus* tissue is intriguing and it will be interesting to know whether it is present in this plantation tree elsewhere in South America, as well as other parts of the world.

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Table 1: Strains of *Enterobacter cowanii* included in this study, LMG = BCCM/LMG Bacteria Collection, Ghent University, BCC = Bacterial Culture Collection, Forestry and Agricultural Biotechnology Institute, Pretoria, South Africa. ^{T} = type strain

Species	Strain	Host	Location
Enterobacter cowanii	LMG 23569^{T} = CCUG 45998^{T} = CIP 107300^{T}	Blood culture	Japan
	BCC 008 = R-25669	*Eucalyptus*	Uruguay
	BCC 009 = R-25670	*Eucalyptus*	Uruguay
	BCC 011 = R-25672	*Eucalyptus*	Uruguay
	BCC 074 = R-21554	*Eucalyptus*	Uruguay
	BCC 078 = R-25680	*Eucalyptus*	Uruguay

Figure 1: Maximum likelihood tree based on complete 16S rRNA sequences of *Enterobacteriacae* species. The tree was generated by the Phyml software using the general time reversible (GTR) model as selected by Modeltest. Bootstrap values after 1000 replicates are expressed as percentages. *Serratia marcescens* was included as an outgroup.

LMG 7871^T|*Kluyvera ascorbata*|AF008579
38
LMG 7870^T|*Raoultella planticola*|AB004756
35
LMG 2784^T|*Enterobacter amnigenus*|AB004749
21
54
LMG 10245^T|*Enterobacter nimipressuralis*|Z96077
23
DSM 30039^T|*Citrobacter freundii*|AJ233408
ATCC-BAA 260^T|*Enterobacter kobei*|AJ508301
ATCC 35953^T|*Enterobacter asburiae*|AB004744
18
59
LMG 2693^T|*Enterobacter cancerogenus*|Z96078
9
LMG 5739^T|*Enterobacter gergoviae*|AB004748
51
ATCC 49851^T|*Enterobacter pyrinus*|AJ010486
CIP 103441^T|*Enterobacter hormaechei*|AJ508302
BCC 078|*Enterobacter cowanii*|EU629164
14
BCC 009|*Enterobacter cowanii*|EU629163
79
45
CCUG 45998^T|*Enterobacter cowanii*|AJ508303
27
D5/23^T|*Enterobacter radicincitans*|AY563134
ATCC 13313^T|*Shigella dysenteriae*|X96966
7
LMG 23730^T|*Enterobacter turicensis*|DQ273681
23
67
27
LMG 2024^T|*Erwinia amylovora*|Z96088
33
LMG 23732^T|*Enterobacter helveticus*|DQ273688
41
ATCC 51329^T|*Enterobacter sakazakii*|AY752937
|*Salmonella enterica*|AF332600
ATCC 13047^T|*Enterobacter cloacae*|AJ251469
LMG 2095^T|*Klebsiella pneumoniae*|AB004753
LMG 2792^T|*Serratia marcescens*|AJ233431

0.1

Figure 2: Maximum likelihood tree based on partial *rpoB* sequences of *Enterobacter* strains. The tree was generated by the Phyml software using the Tamura-Nei (TN93) model as selected by Modeltest. Bootstrap values after 1000 replicates are expressed as percentages. *Serratia marcescens* was included as an outgroup.

BCC 078|*Enterobacter cowanii*|EU629169
BCC 074|*Enterobacter cowanii*|EU629168
81
BCC 008|*Enterobacter cowanii*|EU629165
100
BCC 011|*Enterobacter cowanii*|EU629167
CIP 107300^{T}|*Enterobacter cowanii*|AJ566944
74
78
BCC 009|*Enterobacter cowanii*|EU629166
CIP 108468^{T}|*Enterbacter radicincitans*|AJ783915
93
LMG 23732^{T}|*Enterobacter helveticus*|DQ779997
73
LMG 23730^{T}|*Enterobacter turicensis*|DQ779998
45
LMG 5740^{T}|*Enterobacter sakazakii*|DQ836224
ATCC 49162^{T}|*Enterobacter hormaechei*|AJ543724
47
81
ATCC BAA-260^{T}|*Enterobacter kobei*|AJ566947
48
ATCC 23373^{T}|*Enterobacter dissolvens*|AJ543725
76
67
ATCC 13047^{T}|*Enterobacter cloacae*|AJ543726
72
ATCC 35953^{T}|*Enterobacter asburiae*|AJ543727
ATCC 33241^{T}|*Enterobacter cancerogenus*|AJ566943
65
ATCC 9912^{T}|*Enterobacter nimipressuralis*|AJ566948
100
ATCC 33072^{T}|*Enterobacter amnigenus*|AJ566942
LMG 22970^{T}|*Enterobacter pyrinus*|DQ836223
77
ATCC 33028^{T}|*Enterobacter gergoviae*|AJ566945
ATCC 13880^{T}|*Serratia marcescens*|U77449
0.1

Conclusions

Taxonomy and characterization of species belonging to the former *Erwinia herbicola-Enterobacter agglomerans* complex has become difficult due to the continuous rearrangement of species within the predominantly plant-pathogenic genera *Erwinia*, *Enterobacter*, *Pantoea*, *Pectobacterium*, *Brenneria* and *Dickeya*. The taxonomic issues within these genera have resulted in difficulties with identification and classification and consequently numerous mis-identified and unidentified strains, especially in the genus *Pantoea*. There are very few unique phenotypic differences between genera of the plant-pathogenic *Enterobacteriaceae* and the species belonging to these genera share exceedingly high 16S rRNA sequence similarity. Therefore, a technique was required which could differentiate between *Pantoea* species and closely-related *Enterobacteriaceae*, be used for rapid identification of *Pantoea* strains and hopefully resolve several taxonomic issues within the genus.

Multilocus sequence analysis (MLSA) was selected as the technique of choice for a taxonomic evaluation of the genus *Pantoea*. An MLSA scheme was developed based on the four housekeeping genes *rpoB*, *atpD*, *gyrB* and *infB*. These four housekeeping genes were found to be reliable genetic markers for the identification and classification of *Pantoea* species. In all phylogenetic trees constructed, the seven species of *Pantoea* could be clearly delineated and ten potential new species identified. Furthermore, the MLSA scheme revealed a phylogenetic division of the genus *Pantoea* into the core species and the "Japanese" species, namely *P. citrea*, *P. punctata* and *P. terrea*, and improved our understanding of the relationships of *Pantoea* with its closest phylogenetic neighbours. MLSA has demonstrated the ability to overcome the inconsistencies observed in 16S rRNA phylogeny. In this study, a concatenated data set based on several conserved protein-encoding genes has proven to be a robust and reliable means to resolve taxonomically complex groups of bacteria.

One of the primary objectives of this study was to conclusively identify *Pantoea*-like strains isolated from *Eucalyptus* trees showing symptoms of bacterial blight and dieback in Argentina, Colombia, Uruguay and Uganda and from maize infected with brown stalk rot in South Africa. In a previous study, based on AFLP analysis, it was observed that these strains belonged to the genus *Pantoea*. However, the AFLP clusters observed were not consistent with 16S rRNA sequencing data. A phylogenetic tree based on the concatenated sequences of the four housekeeping genes used in the MLSA scheme clearly differentiated between three well-supported clusters of strains from *Eucalyptus* and maize, and the *Pantoea* core species of *P. agglomerans*, *P. ananatis*, *P. stewartii* and *P. dispersa*. A high correlation was observed between the MLSA data and the DNA-DNA hybridization data of the three clusters. Using MLSA as a supporting technique, four novel species were proposed: *P. vagens* sp. nov. for strains isolated from *Eucalyptus* and maize, *P. eucalypti* sp. nov. and *P. deleyii* sp. nov. for strains isolated only from *Eucalyptus* and *P. anthophila* sp. nov. for strains belonging to protein profile group VII (Beji *et al*., 1988) which were previously allocated to *P. agglomerans*.

The latest edition of Bergey's Manual of Systematic Bacteriology brought attention to four DNA hybridization groups of clinical strains belonging to the former *Erwinia herbicola-Enterobacter agglomerans* complex, which were never assigned to a genus nor described as novel species (Grimont and Grimont, 2005). It was proposed that DNA hybridization groups I, II, IV and V (Brenner *et al*., 1984) should be transferred to the genus *Pantoea* as novel species. Based on this suggestion, strains from DNA hybridization groups II, IV and V were included in the MLSA scheme. Three well-supported clusters grouped closely with the *Pantoea* core species in a concatenated phylogenetic tree, correlating to the three DNA hybridization groups. Additionally, it was noted that the cluster corresponding to DNA hybridization group V contained two sublineages. DNA-DNA hybridization was used to confirm the existence of two novel species within DNA hybridization group V. Consequently, four novel clinical *Pantoea* species were proposed: *P. septica* sp. nov., *P. eucrina* sp. nov., *P. brenneri* sp. nov. and *P. conspicua* sp. nov.

It has been stated that the genus *Pantoea* can be divided into two groups of species: the core group of *P. agglomerans*, *P. ananatis*, *P. stewartii* and *P. dispersa* and the

"Japanese" group of *P. citrea*, *P. punctata* and *P. terrea* (Grimont and Grimont, 2005). This phylogenetic division of *Pantoea* was also observed in the concatenated MLSA tree (Chapter 3). The "Japanese" *Pantoea* species are morphologically and metabolically different to the *Pantoea* core species, and were described based on phenotypic tests and DNA-DNA hybridization data. In the concatenated MLSA tree, the "Japanese" *Pantoea* species formed a tight, well-supported cluster with the type strain of *Tatumella ptyseos* suggesting an affiliation of these species. DNA-DNA hybridization confirmed the close phylogenetic relationship of *T. ptyseos* to the "Japanese" species. The DNA-DNA similarity values between *T. ptyseos* and the "Japanese" species were considerably higher than those between the "Japanese" species and *P. agglomerans* or *P. dispersa*. The "Japanese" species were also found to be more metabolically similar to *Tatumella* than to *Pantoea*. Therefore, it was proposed to transfer the "Japanese" *Pantoea* species to the genus *Tatumella* emended as *Tatumella citrea* comb. nov., *Tatumella punctata* comb. nov. and *Tatumella terrea* comb. nov. The MLSA data also revealed the presence of two species within the *T. citrea* cluster: the *T. citrea* type strain and strains causing pink disease of pineapple. DNA-DNA hybridization validated the existence of two species within *T. citrea*. The strains causing pink disease of pineapple were described as a novel species, *Tatumella morbirosei* sp. nov.

Several non-pigmented, slime-producing strains were isolated simultaneously with *Pantoea* strains from *Eucalyptus* showing symptoms of bacterial blight in Uruguay. The *Pantoea* strains were later proposed as three novel species (Chapter 4). The non-pigmented, slime-producing strains showed highest 16S rRNA sequence similarity to *Enterobacter cowanii*, a primarily clinical species. *rpoB* sequencing placed the strains in a well-supported cluster with the type strain of *E. cowanii* and DNA-DNA hybridization was used to confirm their identity. Pathogenicity tests revealed that *E. cowanii* is not the causal agent of bacterial blight in Uruguay. This is the first report of *E. cowanii* being isolated as an endophyte from *Eucalyptus*.

The MLSA scheme developed for *Pantoea* species raised some questions regarding the relationship of the genus to phylogenetically-related members of the *Enterobacteriaceae*. The genus *Pantoea* appears to be more closely-related to the genera *Tatumella* and *Erwinia* than to the remaining plant-pathogenic genera

Pectobacterium, *Brenneria* and *Dickeya*. However, is has been observed in a previous study that the plant pathogen *Pectobacterium cypripedii* may be associated with the genus *Pantoea* (Young and Park, 2007). The same observation was made in this study when additional phytopathogenic enterobacteria were added to the phylogenetic trees (data not shown). Strains of *Pe. cypripedii* clustered on the border of the genus *Pantoea* with *P. dispersa* and *P. eucrina* in the MLSA phylogenetic trees, distant to the remaining species of the genus *Pectobacterium*. It was also observed in the MLSA trees, that three *Erwinia* species (*E. mallotivora*, *E. psidii* and *E. tracheiphila*) clustered slightly distant to the type species, *E. amylovora* (data not shown). To further investigate the relationship between *Pe. cypripedii* and *Pantoea*, and the possible division within the genus *Erwinia*, more strains should be added to the MLSA scheme to generate stable clusters.

There are still numerous misidentified or unidentified *Pantoea* strains in culture collections and consequently wrongly named sequences in comparison databases. These strains and sequences should classified correctly using MLSA, in order to further improve the rapid identification of environmental *Pantoea* species. Because the genus *Pantoea* is ubiquitous, it is highly likely that many more novel species exist in nature. The sampling strategy should be expanded to include a wider host range and geographical area which should ensure the isolation of *Pantoea* species. The more species and strains which can be added to the MLSA scheme, the clearer the phylogeny of the genus *Pantoea* within the *Enterobacteriaceae* will be.