Bacterial Blight and Dieback of *Eucalyptus* Species, Hybrids, and Clones in South Africa

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ABSTRACT

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During 1998, a new disease appeared on trees representing a *Eucalyptus grandis* × *E. nitens* (GN) hybrid in a nursery in KwaZulu/Natal. The disease has subsequently spread to other *Eucalyptus* species, hybrids, and clones in nurseries and plantations throughout South Africa. Typical symptoms of the disease include dieback of young shoots and leaf blight. This ultimately leads to stunting of trees. The objective of this study was to isolate and identify the causal agent of the disease. A bacterium was consistently isolated from infected tissue. Pathogenicity tests were undertaken with a range of bacterial strains. Four pathogenic strains were selected from different geographical regions and *Eucalyptus* hosts for further study. The bacterium causing *Eucalyptus* leaf and shoot blight is gram negative and rod-shaped, varying in size from 0.5 to 0.75 µm wide and 1.0 to 2.0 µm long. Colonies of this bacterium have a yellow pigment. The results from the Biolog tests identified the bacterium as *Pantoea agglomerans* with a similarity index of 0.315. The 16S rDNA sequences of the purported *Pantoea* sp. were compared with those of other related *Enterobacteriaceae* from GenBank/EMBL. Phylogenetic analysis using PAUP revealed that the isolates group together with *P. agglomerans, P. ananatis,* and *P. stewartii* subsp. *stewartii*. The fatty acid profiles and phenotypic characteristics of the new pathogen are similar to *P. ananatis*, and % G + C is within the range of this species. DNA:DNA hybridization between the four strains and the type strain of *P. ananatis* conclusively showed that the bacterium causing blight and dieback of *Eucalyptus* in South Africa belongs to this species. This is the first report in which *P. ananatis* has been found as a causal agent of a disease on *Eucalyptus*.

Additional keywords: *Erwinia ananas, Erwinia uredovora*

In South Africa, *Eucalyptus* species, hybrids, and clones are grown commercially and account for more than 50% of all newly afforested areas (1). This hardwood tree is used for the production of solid timber products and is also the basis of an internationally important pulp and paper industry. A number of fungal diseases cause severe damage to eucalypts in South Africa, notably Cryphonectria canker (4), Coniothyrium canker (26), Botryosphaeria canker (19,20), and Mycosphaerella leaf blotch (6). Thus far, only two bacterial diseases have been reported on this host, namely bacterial wilt caused by *Ralstonia solanacearum* (7) and bacterial dieback caused by *Xanthomonas eucalypti* (22). Bacterial wilt has recently been reported for the first time on *Eucalyptus* in South Africa (5), while in Australia bacterial dieback has been found only on *Eucalyptus citriodora* (22).

In 1998, a severe disease appeared in a single nursery in KwaZulu/Natal, South Africa, on ramets of an *E. grandis* × *E. nitens* (GN) hybrid clone. The disease subsequently spread to other nurseries and commercial plantations and to a number of different *Eucalyptus* species, hybrids, and clones. Typical symptoms of bacterial blight (Fig. 1) include tip dieback and leaf spots on young leaves. The leaf spots are initially water-soaked and often coalesce to form larger lesions. The pathogen appears to spread from the leaf petiole into the main leaf vein and from there to the adjacent tissue. Thus, lesions on the leaf are often concentrated along the main veins. Leaf petioles become necrotic, which results in premature abscission of the leaves. Trees assume a scorched appearance in the advanced stages of the disease and after repeated infections become stunted. Due to the resultant formation of many new growing tips and epicormic shoots, the trees have a bushy appearance. Highly susceptible species, hybrids, and clones exhibit a combination of dieback and blight symptoms, while those more tolerant show only leaf spot symptoms. A bacterium was consistently isolated from symptomatic tissue and tentatively identified as an *Erwinia* sp. based on morphology, motility, colony color, fermentative utilization of glucose, and results from catalase and oxidase tests.

The disease on *Eucalyptus* is more prevalent in areas of South Africa where the temperatures are relatively low (between 20 and 25°C) and the relative humidity high. The means of entry of this pathogen into its host has yet to be established. However, in nurseries where vegetative propagation is practiced, the bacterium enters the cut surfaces of cuttings and reduces their ability to root by nearly 100%.

Eucalyptus spp. belong to the family Myrtaceae. The only member of this family that is reported to be affected by an *Erwinia* sp. is guava (*Psidium guajava*). In 1987, a previously undescribed bacterial disease caused severe losses of guava trees in Brazil (16). The pathogen attacked branches and twigs, causing severe dieback and ultimately death of trees. The pathogen was identified as *Erwinia psidii* sp. nov. Pathogenicity tests were undertaken with different members of the Myrtaceae. *E. citriodora* and *E. saligna* were included but were found not be hosts of this pathogen.

The objective of this study was to identify the causal agent of bacterial blight and dieback affecting important *Eucalyptus* species, hybrids, and clones in South Africa. Pathogenicity tests were undertaken, and the causal agent was characterized both phenotypically and genotypically using a variety of techniques.

MATERIALS AND METHODS

Isolation procedures. Plant material showing typical symptoms of the disease

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was collected from nurseries and commercial plantations of *Eucalyptus* in South Africa. Leaves with distinct water-soaked lesions, as well as wilted shoots, were surface-sterilized in a 10% solution of commercial bleach (5.25% sodium hypochlorite), washed with sterile distilled water, and then placed in a sterile mortar containing 1 ml of sterile distilled water. The leaves were crushed with a sterile pestle, and the resulting suspension was streaked onto nutrient agar (Biolog, Hayward, CA). The cultures were incubated at 30°C for 3 days.

Pathogenicity tests. Pathogenicity tests were conducted on an *E. grandis* × *E. nitens* (GN) clone found to be highly susceptible to the bacterial blight pathogen in nurseries and plantations. Twenty 3-month-old plants were inoculated with each bacterial strain in two separate experiments. Two inoculation methods were used: a fine needle was dipped into a bacterial suspension (2×10^6 CFU/ml) and then gently inserted into the surface of young leaves (four wounds were made per leaf) and into the petioles of young leaves. Control plants were inoculated with sterile water. Plants were incubated in a controlled environment chamber (Controlled Environments Ltd., Winnipeg, Manitoba, Canada) at temperatures between 20 and 23°C and relative humidity between 80 and 90%. The plants were inspected daily. Once symptoms were expressed, bacteria were reisolated from infected leaves using the technique described previously. The reisolated strains were compared with the inoculated strains using selected phenotypic tests (morphology, Gram stain, colony morphology, Hugh-Leifson, oxidase, and catalase tests).

Nutritional and physiological tests. Once pure cultures of the bacterial strains were obtained, characteristics such as cell morphology, Gram stain, colony morphology, and flagellar arrangement were determined. The Hugh-Leifson oxidation/fermentation, oxidase, and catalase tests

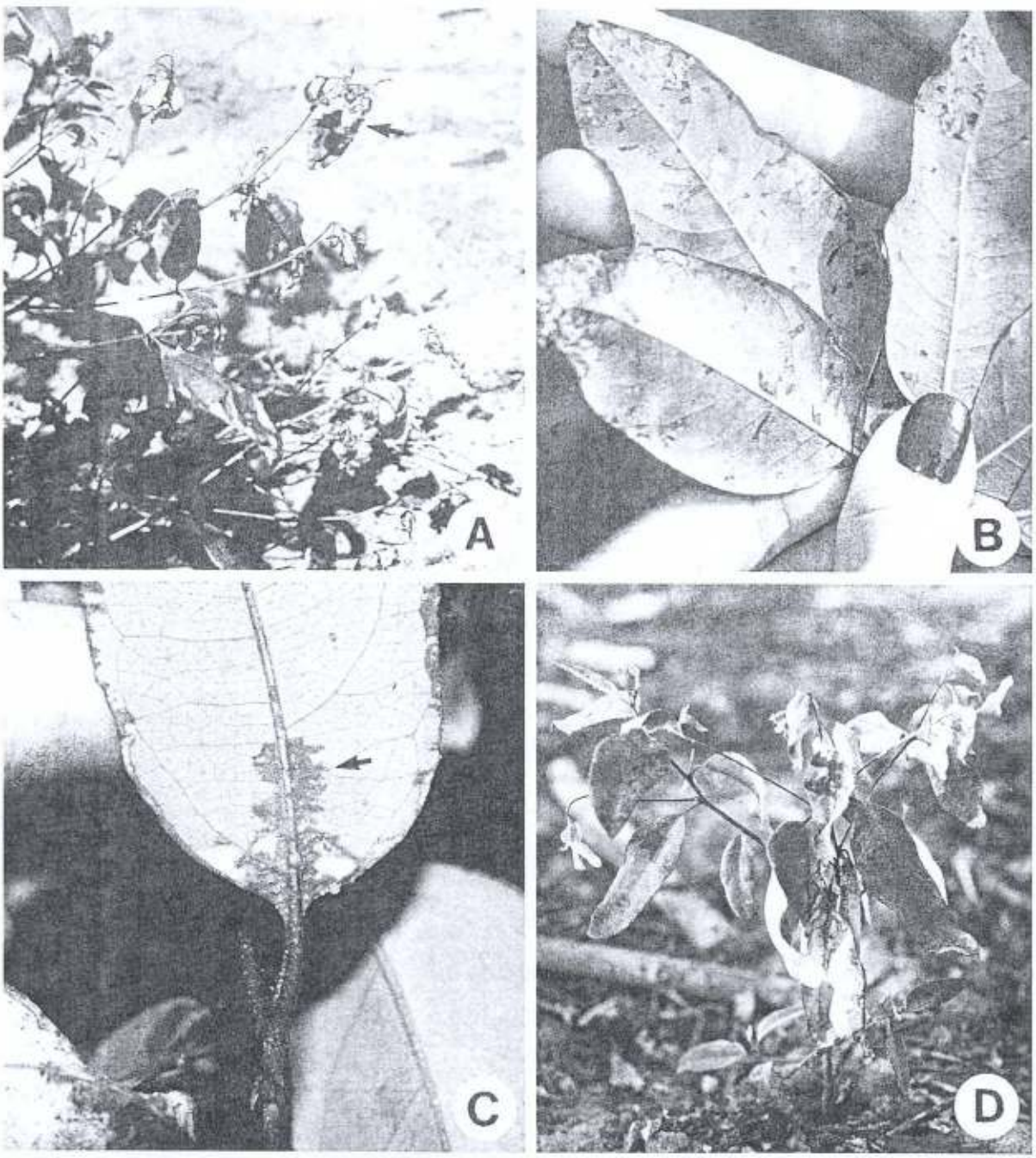

Fig. 1. Symptoms associated with bacterial blight and dieback of *Eucalyptus*. **A,** Typical dieback symptoms (arrow). **B,** Water-soaked spots on leaves. **C,** Lesions coalescing along the main vein of the leaf (arrow). **D,** Scorched leaves and shoots.

also were conducted. The bacterial strains were identified using Biolog's Gram negative GN Microplate technique, together with Biolog's MicroLog version 4.0 software (Biolog).

Tests forming part of the API 20E and API 50 CHE systems (BioMérieux, La Balme les Grottes, Montalieu Vercieu, France) were performed on the four strains from *Eucalyptus* and the type strain of *Pantoea ananatis*, LMG 2665^T, using the procedure described by Mergaert et al. (14). Acid production from carbohydrates and esculin hydrolysis was recorded after 48 h using the API 50 CHE tests. Gelatin liquefaction, indole acetoin, and hydrogen sulfide production, arginine dihydrolase, lysine, and ornithine decarboxylases, tryptophan deaminase, ß-D-galactosidase, urease, and citrate utilization were recorded after 24 h using the API 20E tests. Profiles were identified using the APILAB V4.0 identification program (BioMérieux).

Fatty acid analysis. The strains from *Eucalyptus* and LMG 2665^T were characterized by the profiles of fatty acid methyl ester separated by gas-liquid chromatography. The profiles were compared with those of known species in the Microbial Identification System (MIS) database, TSBA version 4.0 (MICROBIAL ID Inc., Newark, DE).

16S rRNA gene amplification and sequencing. A section of the 16S rRNA gene of the four strains was amplified using the universal eubacterial primer pairs, fD1 (5′-AGA GTT TGA TCC TGG CTC AG-3′) and rD1 (5′-AAG GAG GTG ATC CAG CCG CA-3′) (25). The purified polymerase chain reaction (PCR) products were cloned and then sequenced with the primers fD1 and rD1 as well as with the internal primers OT1 (5′-GAA GAA GGC CTT CGG GTT G-3′) and OT2 (5′-CAC GAC ACG AGC TGA CGA C-3′) (25) using the Big Dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). Sequences were analyzed using an ABI Prism 377 DNA sequencer and edited with the program Sequence Navigator (Applied Biosystems). The GenBank/EMBL databases were used for homology searches using the BLAST program (National Center for Biotechnology Information, U.S. National Institutes of Health, Bethesda, MD). A selection of 16S rDNA sequences obtained from a BLAST search were aligned with the partial sequences of the four strains using CLUSTALW (21). The alignment was trimmed of the overhangs before parsimony analysis. Heuristic searches with maximum parsimony used stepwise (simple) addition and tree-bisection-reconnection to produce a phylogenetic tree (PAUP 4.0b3, D. L. Swofford, Illinois Natural History Survey, Champaign). Bootstrap values were obtained from the same data matrix.

DNA base composition determination. The strains from *Eucalyptus* and type strain LMG 2665^T were grown on nutrient agar (Oxoid, Basingstoke, Hampshire, England) at 28°C. DNA was extracted from these isolates using the method described by Pitcher et al. (17). DNA base composition was determined using the high-pressure liquid chromatography (HPLC) method. DNA was enzymatically degraded into nucleotides using the method described by Mesbach et al. (15). The obtained nucleotide mixture was then separated by HPLC using a Waters Symmetry Shield C8 column at 37°C. The solvent used was 0.02 M $NH_4H_2PO_4$ (pH = 4.0) with 1.5% acetonitrile. Nonmethylated lambda phage DNA (Sigma Chemical Co., St. Louis, MO) was used as the calibration reference.

DNA-DNA hybridizations. DNA-DNA hybridizations were performed with photobiotin-labeled probes in microplate wells as described by Ezaki et al. (8). A HTS7000 BioAssay Reader (Perkin-Elmer Cetus, Norwalk, CT) was used for the fluorescence measurements. The hybridization temperature was 40°C. Reciprocal experiments were performed for every pair of strains, and the means from reciprocal tests are presented.

RESULTS

Isolation procedures. Bacteria were consistently isolated from infected leaves. No bacteria were isolated from healthy tissue. For characterization and pathogenicity tests, four strains from different *Eucalyptus* hosts and geographic regions were used (Table 1). Strains LMG 20103, LMG 20104, LMG 20105, and LMG 20106 were deposited in the BCCM/LMG Culture Collection (Universiteit Gent, Belgium).

Pathogenicity tests. Water-soaked lesions developed on leaves of all plants inoculated with all four strains 3 days after inoculation. After 10 days, the lesions were necrotic and in some cases had coalesced. After 14 days, large necrotic lesions had developed on the leaves, and there appeared to be no progression of symptoms from these lesions into the main vein and petioles of the host plant. When leaf petioles were inoculated with the pathogen, tissue adjacent to the main vein became water-soaked after 6 days, and after 14 days the leaves wilted, became necrotic, and abscised from the plant. A *Pantoea* sp. was reisolated from lesions on the leaves and from main veins using the method described previously. Control plants showed no symptoms. The *Pantoea* sp. isolated from these inoculated plants was shown to be identical, based on phenotypic characteristics, to the original strains used in these pathogenicity tests.

Nutritional and physiological tests. The strains isolated from *Eucalyptus* species, hybrids, and clones showing typical symptoms of bacterial blight and dieback were Gram negative, rod-shaped, 0.5 to 0.75 µm in width and 1.0 to 2.0 µm in length with a peritrichous flagella arrangement. All strains were yellow pigmented. The strains fermentatively utilized glucose and were oxidase negative and catalase positive. Computer analysis of the

Table 1. Bacterial strains from *Eucalyptus* characterized in this study

Strain Number	Origin	Host
LMG 20103	Piet Retief, Mpumalanga	*E. grandis* × *E. nitens* hybrid A
LMG 20104	Harding, KwaZulu/Natal	*E. grandis* Clone A
LMG 20105	Pietermaritzburg, KwaZulu/Natal	*E. grandis* × *E. nitens* hybrid B
LMG 20106	Tzaneen, Northern Province	*E. grandis* Clone B

Table 2. Fatty acid compositions of bacterial strains from *Eucalyptus* and *Pantoea ananatis* LMG 2665^T

	Percentage of total	
Feature[z]	Bacterial strains from *Eucalyptus* (range for four isolates)	*Pantoea ananatis* LMG 2665^T (average of five replicates)
12:0	4.7 to 5.8	3.8
14:0	1.8 to 3.1	2.6
15:0	0.4 to 1.9	1.6
14:0 2OH	2.9 to 4.6	2.4
16:0	24.2 to 29.3	29.3
17:0 cyclo	3.4 to 4.5	8.0
17:0	0.5 to 2.8	2.7
17:0 10 methyl	0.3 to 2.8	0.6
18:1 ω7c	13.5 to 16.8	17.6
Summed feature 2	10.3 to 15.2	8.5
Summed feature 3	22.5 to 27.0	20.1

[z] Also found in some or all isolates in trace amounts (≤1% of total): 10:0 3OH, 13:0, 12:0 2OH, 12:0 3OH, unknown 13.957, unknown 14.502, 15:0 anteiso, 16:0 N alcohol, 17:1 ω8c, 16:0 3OH, 18:0, 19:0 cyclo, summed feature 1. Summed feature 1 comprises any combination of 15:1 iso H, 15:1 iso I, and 13:0 3OH. Summed feature 2 comprises any combination of 12:0 aldehyde, unknown 10.928, 16:1 iso I, and 14:0 3OH. Summed feature 3 comprises 15:0 iso 2OH, 16:1 ω7c, or both.

data using the Biolog Gram negative microplate system showed that the strains belong to the *Enterobacteriaceae*. Although the Biolog system gave no exact identification, as reflected by the low percent similarity, the metabolic profile of the bacterial strains most closely resembled the database profile of *Pantoea agglomerans*, with a similarity index of 0.315.

Strains LMG 20103, LMG 20104, LMG 20105, and LMG 20106 and *Pantoea ananatis* strain LMG 2665^T were positive for acid production from glycerol, L-arabinose, ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, *meso*-inositol, mannitol, sorbitol, *N*-acetylglucosamine, arbutine, salicine, cellobiose, maltose, lactose, melibiose, saccharose, trehalose, D-raffinose, D-arabitol, esculine hydrolysis, ß-galactosidase, citrate utilization, indole production from tryptophan, and acetoin production. The strains were negative for acid production from *meso*-erythritol, D-arabinose, L-xylose, adonitol, ß-methylxyloside, L-sorbose, dulcitol, α-methyl-D-glucoside, inuline, melezitose, starch, glycogen, *meso*-xylitol, D-turanose, D-lyxose, D-tagatose, D- and L-fucose, L-arabitol, gluconate, 2-keto-gluconate, 5-keto-gluconate, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, hydrogen sulfide production, and tryptophan deaminase. Different reactions, positive or negative, were obtained for urease and α-methyl-D-mannoside, amygdalin, and D-gentiobiose.

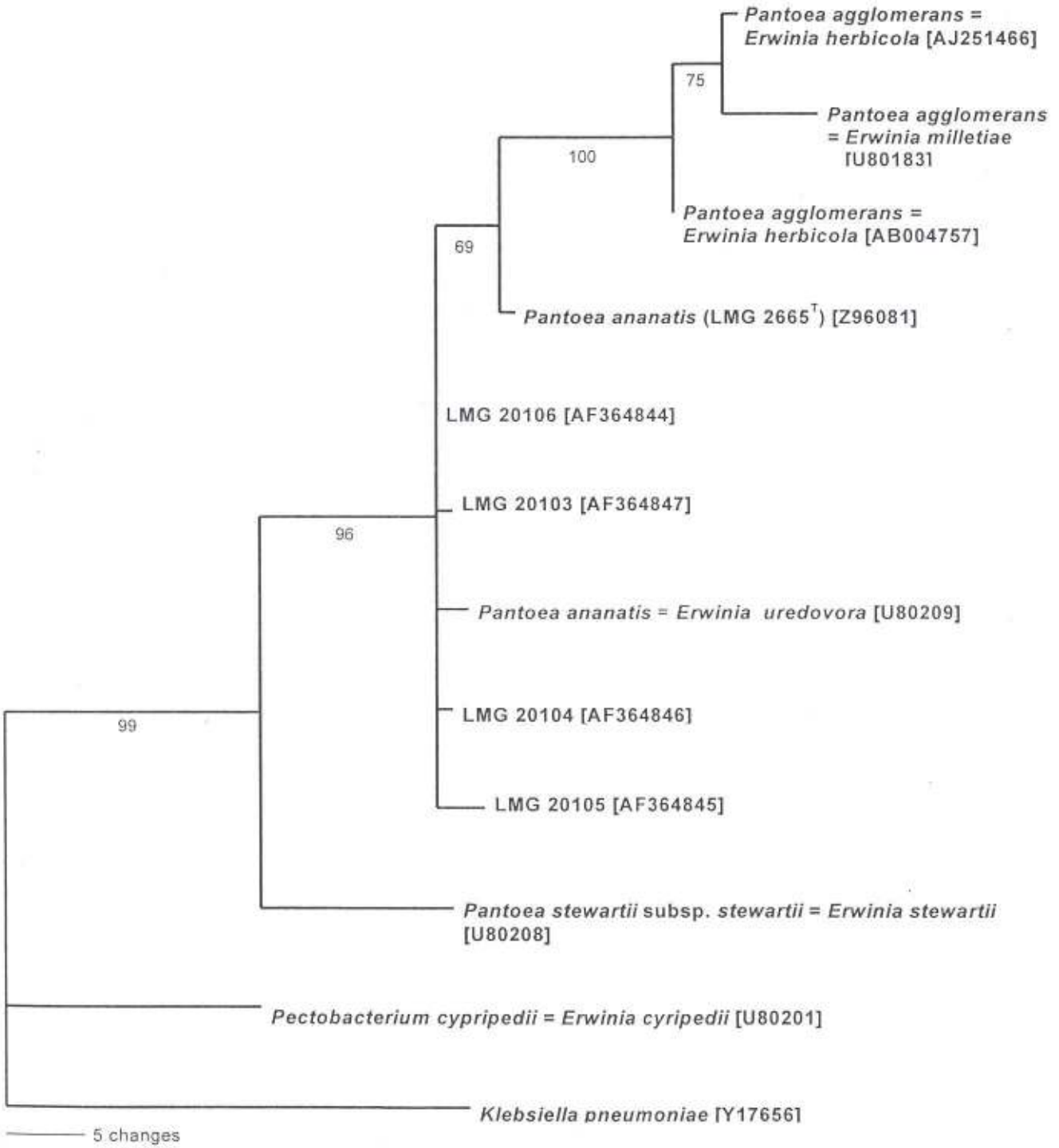

Fig. 2. One of seven most parsimonious trees of 114 steps based on selected partial 16S rDNA sequences from *Pantoea* species, including the four strains from *Eucalyptus*. The sequence of *Klebsiella pneumoniae* was used as an outgroup taxon. Bootstrap values are marked below the branches. Accession numbers of the sequences obtained from the GenBank/EMBL databases have been placed in brackets.

Unlike *Pantoea ananatis* LMG 2665^T, the strains from *Eucalyptus* liquefied gelatin. Urease was reported to be negative for all 18 strains of *P. ananatis* studied by Mergaert et al. (13), while gelatinase and acid production from α-methyl-D-mannoside, amygdalin, and D-gentiobiose differed among *P. ananatis* strains. The profiles of the strains from *Eucalyptus* were not recognized unambiguously by the API 20E recognition program.

Fatty acid analysis. The fatty acid compositions of the *Eucalyptus* strains were very similar to that of *P. ananatis* LMG 2665^T (Table 2) and to those of 18 *P. ananatis* strains as reported by Mergaert et al. (13). The MIS identification program identified the *Eucalyptus* strains as *Cedecea davisae* (maximum score 0.583), and *P. ananatis* was the second most similar (maximum score 0.474).

16S rDNA sequence analysis. Partial 16S rDNA sequences of the four suspected *Erwinia* spp. were obtained and deposited into the GenBank database with accession numbers AF364844 (LMG 20106), AF364845 (LMG 20105), AF364846 (LMG 20104), and AF364847 (LMG 20103). With the exception of a few nucleotide differences, the four sequences were identical. A BLAST search with the 1.5-kb 16S rDNA sequences of the four strains supported the Biolog results that the isolates belong to a species of *Pantoea*. The highest homologies were to the 16S rDNA of *E. uredovora* (accession number U80209) and of *P. ananatis* LMG 2665^T (accession number Z96081) with identities of 99.7 and 99.0%, respectively. Seven most parsimonious trees were produced from the analysis of the aligned sequences of the 1.3-kb 16S rDNA sequences generated in this study and of other *Enterobacteriaceae* (Fig. 2). Of the 1,295 characters used, 46 of the variable characters were parsimony uninformative and 44 parsimony informative. The total tree length was 114 steps, consistency index was 0.877, and the retention index was 0.825. The trees show clearly that the sequences for the isolates in this study group into a cluster with *P. ananatis* (syn. *Erwinia uredovora*).

DNA base composition determination and DNA-DNA hybridizations. The DNA base ratios for the bacterial strains from *Eucalyptus* were 52.8 to 53.9 mol % G+C and for *P. ananatis* LMG 2665^T were 53.3 mol % G+C. The DNA-DNA binding ratios among the bacterial strains from *Eucalyptus* and *P. ananatis* LMG 2665^T were between 94 and 103%, with an average SD of 12%, which is slightly above the SD of 7% mentioned by Goris et al. (11).

DISCUSSION

In this study we report, for the first time, on the occurrence of bacterial blight and dieback initiated by *P. ananatis* on *Eucalyptus* in South Africa. Koch's postulates were confirmed using four strains, and these were subjected to further taxonomic investigation. Phenotypic characterization of the strains supported our preliminary results that the causal agent of the disease is a *Pantoea* sp. The genus *Pantoea* was established in 1989 and includes plant-pathogenic species formerly classified as *Erwinia herbicola*, *E. milletiae* (9), *E. ananas*, *E. uredovora*, and *E. stewartii* (13). This is the first report of a *Pantoea* sp. infecting *Eucalyptus*. It also records a serious new disease problem affecting one of the most widely planted forest trees in South Africa and elsewhere in the world.

The genotypic characterization of the four strains from *Eucalyptus* confirmed that the causal agent is *P. ananatis* (synonyms, *Erwinia ananas* and *E. uredovora*, 13). Hauben et al. (12) showed, using 16S rDNA sequences of 29 plant-associated strains, representing *Erwinia*, *Pantoea*, and other *Enterobacteriaceae*, that three phylogenetic groups exist within *Pantoea*. *Pantoea* spp. group in a monophyletic unit (Cluster IV) that is closely related to the true *Erwinia* species. The values obtained for the DNA base ratios of the four strains were similar to the 53.1 to 55.2% reported for 10 authentic strains of *P. ananatis* (13). The DNA binding ratios between the isolates from *Eucalyptus* and *P. ananatis* LMG 2665^T were higher than 94%. The microplate method used in this study has been shown to be well correlated to the initial renaturation method used by Mergaert et al. (13), who found 76% as the lowest binding value among strains classified as *P. ananatis*. Morphological, nutritional, and physiological characteristics and fatty acid compositions were very similar to those of *P. ananatis* LMG 2665^T and those reported for 18 strains of this species (13). We thus conclude that the bacterium associated with *Eucalyptus* leaf blight and dieback belongs to this species.

P. ananatis was first reported as a pathogen of pineapple fruitlets, causing brown rot (18). However, there has been no supplementary evidence of this disease on this host since the original report in 1928. Sudangrass, cantaloupe fruit, sugarcane, onions, and honeydew melons have also been reported as hosts of this pathogen (2,3,8,10,24). In a recent study by van Zyl (23), two *Pantoea* spp. were found to exist in a synergistic relationship with a fungal pathogen of *Eucalyptus*, *Coniothyrium zuluense*, which causes a serious canker disease of this host. Using 16S rRNA gene sequence comparisons, one of the *Pantoea* spp. was identified as *P. ananatis*. The fact that the bacterium found to be responsible for bacterial blight and dieback is the same as that involved in Coniothyrium canker is intriguing and deserves further investigation.

Bacterial blight and dieback has become a serious problem in nurseries and young plantations throughout South Africa. Not only is the bacterium infecting cuttings but also ramets in the nursery. This seriously hinders the ability of forestry companies to produce vegetative material for rooting. *P. ananatis* has the ability to infect a number of *Eucalyptus* clones, hybrids, and species, including *E. grandis*, *E. saligna*, *E. dunnii*, *E. nitens*, *E. smithii*, *E. grandis* × *E. camadulensis* (GC), and *E. grandis* × *E. urophylla* (GU). This is of considerable concern, as these represent some of the most crucial planting stock on which a major forestry industry is based. There are, however, significant differences in susceptibility among *E. grandis* clones, and this provides an excellent opportunity for the selection of tolerant material. Development of management strategies to reduce the impact of this disease is now a priority. A rapid screening technique to detect this bacterium is needed, and commercially important clones should be tested to determine their level of tolerance to this disease.

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