CHAPTER 3

Isolation and Identification of P*antoea ananatis* from Onion Seed in South Africa

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Summary

Center rot of onion, caused by the gram-negative, facultatively anaerobic bacterium, *Pantoea ananatis*, was first described in Georgia, USA, in 1997. The disease was later reported in Colorado and Michigan, but not in any other area of the world. *P. ananatis* is seed-borne and seed-transmitted in onions. A similar disease, leaf and seed stalk necrosis of onion, was reported in South Africa in 1981, and it was suggested that *P. ananatis* was responsible for that disease and was introduced into the USA on infected seed lots from South Africa. However, a representative strain of the leaf and seed stalk necrosis pathogen, isolated in South Africa in 1981, was identified as *P. agglomerans* in this study. As center rot of onion has not been observed in South Africa, and *P. ananatis* was not isolated from diseased plants, the main objectives of this research were to determine if pathogenic *P. ananatis* is present in local onion seed and to compare the South African strains to center rot causing strains from the USA. Colonies resembling those of *P. ananatis* were isolated from four seed lots on a semi-selective medium, PA 20. Pathogenicity tests demonstrated that the South African strains could induce the same symptoms on onion as those caused by the American strains. Physiological and biochemical characterization using Biolog, API 20E and API 50 CHE systems, and analysis of the 16S rRNA gene sequences identified the strains from seed as *P. ananatis*. This is the first report on the presence of the center rot pathogen of onion, *P. ananatis*, in South African onion seed.

Introduction

Center rot disease of onion was first observed in Georgia, USA, on Sweet Vidalia onions in 1997 (Gitaitis and Gay, 1997) and later in Colorado (Schwartz and Otto, 2000a) and Michigan (Mark *et al.*, 2002). The disease has occurred in commercial fields in Georgia every year since 1997 and accounted for 100% loss in some fields (Walcott *et al.*, 2002). The causal agent of center rot is a Gram-negative, facultatively anaerobic bacterium, identified as *Pantoea ananatis* (Gitaitis and Gay, 1997). The disease affects the center leaves of onions, which become water-soaked, soft, and bleached white as the rot progresses. Advanced stages of the disease result in complete wilting and bleaching of all leaves (Mark *et al.*, 2002; Walcott *et al.*, 2002). Walcott *et al.* (2002), reported natural infestation and transmission of *P. ananatis* in onion seed and implied that seed was the primary source of inoculum. This pathogen is known to be seed-borne and seed-transmitted in rice (Tabei *et al.*, 1988), sudangrass (Azad *et al.*, 2000) and buckwheat (Iimura and Hosono, 1996).

In the 2004/2005 growing season 358 tons of onion seed were produced in South Africa, of which 282 tons were for the export market (Sansor Annual Report, 2005). Efforts to control the quality of commercially produced onion seed concentrated on the detection of fungal pathogens, and little is known about bacterial pathogens that may be present. *P. ananatis* is the causal agent of bacterial blight and dieback of *Eucalyptus* in South Africa (Coutinho *et al.*, 2002) but center rot of onion has not been reported. However, in 1981, *Erwinia herbicola* was reported to cause a similar disease, stalk and leaf necrosis of onion in South Africa (Hattingh and Walters, 1981). In 1989, Beji *et al*. (1988) proposed transferring *E. herbicola* (syn. *E. milletiae*) to the new genus *Pantoea* (Gavini *et al*., 1989), that included species formerly classified as *E. ananas*, *E. uredovora* and *E. stewartii* (Mergaert *et al.*, 1993). The seed associated with the first outbreak of center rot of onion in Georgia, USA, was produced in South Africa and Walcott *et al*. (2002) suggested that the center rot pathogen was possibly introduced on infested seed lots.

The goal of this study was to determine if pathogenic *P. ananatis* was present in South African onion seed and to compare such strains to those associated with center rot of onions in the USA.

Materials and methods

Media used for isolations

Two different culture media were used for isolations. The first was a general growth medium, nutrient agar (NA) (Difco, Sparks, MD) as recommended by Walcott *et al.* (2002). Another was a semi-selective medium PA 20 (Goszczynska *et al.*, 2006) developed specifically for isolation of *P. ananatis* from onion seed. PA 20 contained the following per liter: NaCl, 20 g; K_2HPO_4, 1 g; $NH_4H_2PO_4$, 1 g; $MgSO_4x7H_2O$, 0.2 g, bromothymol blue, 1 ml of 1.6% aq. solution; crystal violet, 2 ml of 0.075% aq. solution; agar, 15 g. The pH was adjusted to 8.0 with 1.0 N NaOH. After autoclaving and cooling to 50^o C, 3 g of D (+) arabitol dissolved in 5 ml water and 2 ml of 1% aq solution of thallium nitrate were added. Both solutions were filter sterilized prior to adding them to the medium.

Isolations from plants

Diseased onion fields in the Small Karoo area of the Western Cape Province were identified by the onion seed producers in June-July and November-January between 2001-2004. Disease symptoms on twenty-four experimental fields of *Allium cepa*, Granex type, were similar to leaf and stalk necrosis described by Hattingh and Walters (1981). Irregular oval spots, 4-10 cm long with a necrotic center and a dirty yellow or water-soaked margin lesions were found on onion leaves and seed stalks. Small water-soaked lesions were also present.

In June-July 2003, nineteen onion fields of commercial bulb producers in the Northern and Free State Provinces were inspected. Onions from these fields were healthy.

In April 2005, on one water-logged field in the Northern Province, where the cv. Granex 33 was grown, symptoms similar to that of center rot were observed. The youngest, center leaves of onions were chlorotic, becoming soft and wilted as the disease advanced. A light tan discoloration progressed into bulbs, resulting in the decay of the inner scales. The disease affected approximately 50% of the crop.

Ten to twenty diseased plants were collected from each field and isolations were done from all collected plants. Tissue from lesion margins of infected leaf, seed stalk or bulb was removed with a sterile scalpel and macerated in 1 ml of sterile quarter-strength Ringer buffer (Oxoid, Basingstoke, Hampshire, England) for 20 min at 25^{o}C. Plant extracts were streaked onto NA and PA 20. NA and PA 20 plates were evaluated after four and seven days of incubation at 25^{o}C, respectively. Suspected colonies were purified by streaking onto NA and

cultures stored in milk-glycerol liquid medium (10% skim milk, 15% glycerol in distilled water) at –20°C for further analysis.

Isolations from seed

Thirty-two seed samples were evaluated for the presence of *P. ananatis*: twenty-eight from *A. cepa*, two from *A. fistulosum* and two from *A. porri*. Fifteen *A. cepa* seed samples were commercial seed: six of cv. Granex 33, three of cv. Pegasus, two of cv. Pyramid, one of cv. Capricio and three of experimental onions (Granex type). The seed were produced in the Small Karoo area and harvested one or two years prior to testing. One sample, of an unknown cultivar, was from a farmer in the Northern Province, who produced the seed for his own use. The remaining twelve seed samples were harvested in January 2004 from umbels of experimental onion (Granex type) fields in the Small Karoo, in which leaf blight developed naturally. Twenty umbels were collected from each field and pooled into one sample. Seeds were extracted manually and stored in paper bags at 4°C until processing.

Isolations from seed were carried out as described previously (Goszczynska *et al.*, 2006). For each seed lot, two sub-samples, 5 g each, were crushed and placed into separate sterile Erlenmeyer's flasks containing 100 ml of a quarter-strength Ringer buffer. Flasks were incubated for 30 min at 25°C on a rotary shaker at 180 rpm/min. For each sample, four ten-fold serial dilutions were made in half strength nutrient broth. A volume of 0.1 ml of each dilution was plated onto three plates each of NA and PA 20. Plates were incubated at 25°C. Colonies were counted and evaluated after 7 days. Suspected colonies of the pathogen were purified by streaking onto NA and stored at –20°C in milk-glycerol liquid medium for further analysis.

Preliminary characterization of strains

From each seed lot, 20 yellow colonies from NA and 20 yellow colonies from PA 20 were purified and used in preliminary pathogenicity and biochemical tests. The strains from seed washings were characterized by colony morphology, pigment production on NA, motility, cell morphology, Gram stain, oxidase reaction, catalase reaction, indole production, Hugh-Leifson oxidation/fermentation test, gas production from glucose and hydrogen sulphide production from cysteine (Mergaert *et al.*, 1993), according to methods described by Fahy and Hayward (1983). Pathogenicity on onion was determined in green house assays by using a stub inoculation test (Goszczynska *et al.*, 2005). A sterile needle was dipped into the bacterial colony on NA (24-48 hours growth) and then the needle was inserted under the

epidermis of a leaf. At least two leaves were inoculated per isolate. Inoculated plants were incubated in a greenhouse with 27°C/23°C day/night temperatures and observed daily for the development of symptoms.

Bacterial strains

The following bacterial strains from onion were characterized in the study: three strains isolated from South African seed (Table 1), the strain reported to cause leaf and seed stalk necrosis of onion in 1981 and four *P. ananatis* strains associated with center rot in the USA (Table 2). Two *P. ananatis* strains causing blight of *Eucalyptus,* and the type strains of *P. ananatis* LMG 2665^T and *P. agglomerans* LMG 1286^T were used as reference strains (Table 2). Stock cultures of all strains were maintained in milk-glycerol liquid medium at –20°C. Stock strains were transferred onto NA plates and incubated at 25°C to recover growing cultures. Cultures were routinely checked for purity and colony characteristics on NA.

Pathogenicity tests

Spray inoculations were performed with strains listed in Table 2. Strains were grown on NA at 25°C for 24 hours. Bacterial suspensions were made in sterile distilled water to obtain approximately 10^7 colony forming units (CFU)/ml (as determined by dilution plating on NA). Six to eight week-old onion plants (*A. cepa* - cv. Granex 33 and cv. Pyramid; *A. fistulosum* - cv. White Welsh) and leek plants (*A. porri* - cv. Giant Italian) were spray inoculated to runoff with each bacterial suspension. Negative control plants were inoculated with sterile distilled water. Three plants were inoculated with each strain. Plants were incubated for 72 hours in a humidity chamber at 27°C and relative humidity of 95%. Later plants were maintained in a greenhouse with 27°C/23°C day/night temperatures and observed daily for three weeks for the development of symptoms.

The identities of the bacteria re-isolated from lesions were confirmed by colony morphology on NA and PA 20, Gram stain, oxidase reaction, Hugh-Leifson test, and indole production (Coutinho *et al.*, 2002; Walcott *et al.*, 2002).

Biochemical and physiological tests

Strains listed in Table 2 were characterized by sole carbon source utilization profiles, using Biolog GN Microplates together with Biolog MicroLog version 4.2 software (Biolog, Inc., Hayward, CA), according to the manufacturer's instructions. The strains were also tested with the API 20E and API 50CHE systems (BioMérieux, La Balme les Grottes,

Montalieu Vercieu, France) using the procedure recommended by the manufacturers. The results of the API 20E and API 50CHE tests were recorded after 24 and 48 hours of incubation at 30°C respectively. Profiles were identified using the APILAB V4.0 identification program (BioMérieux).

Amplification and sequencing of the 16S rRNA gene

Genomic DNA of strains was extracted by the cetyltrimethylammonium bromide (CTAB) method according to the DNA Miniprep protocol of Wilson (1989). Colonies from each strain were picked from the 48 hours NA plates and transferred into micro centrifuge tubes with sterile STE (10mM Tris-HCl, 1mM EDTA, 2M NaCl, pH 8.0) buffer. Cell lysis was obtained by incubation with sodium dodecyl sulfate and selective precipitation of cell debris and polysaccharides with CTAB/NaCl. DNA was extracted with phenol-chloroform-isoamyl alcohol, precipitated with isopropanol, washed with ethanol, air dried and dissolved in 100 µl of sterile water. Purified DNA was quantified using a Dyna Quant 200 fluorometer (Hoefer, San Francisco, CA) and Hoescht H 33258 intercalating dye (Polysciences, Warrington, PA). The DNA concentration was adjusted to 25-50 ng μl^{-1} with sterile water.

The 16S rRNA gene fragment was amplified using the universal primers fD1 (5'-AGA GTT TGA TCC TGG CTC AG-3') and rD1 (5'-AAG GAG GTG ATC CAG CCG CA-3') (Weisburg *et al.*, 1991). DNA was amplified in 50 µl reaction volumes containing polymerase chain reaction (PCR) buffer (10 mM Tris-HCl, pH 9.0 at 25°C; 50 mM KCl, 0.1% Triton X-100); 1.5 mM $MgCl_2$; 150 µM dNTPs; 1.0 µM each primer; Taq polymerase (Promega Corp., Madison, WI) 1 unit per reaction volume and 25-50 ng DNA template μl^{-1}. Amplifications were performed according to Weisburg *at al*. (1991) in a Hybaid Omni Gene thermocycler (Teddington, England).

The amplified PCR products were cut from the gel and purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany), and then cloned with the pGEM-T Easy Vector System II kit (Promega, Madison, WI) according to the manufacturer's instructions. Plasmids with inserts were purified using the QIAprep Spin kit (Qiagen). The sequencing was performed using the Dyenamic ET Dye terminator cycle sequencing kit for MegaBACE with dideoxynucletide chain-termination chemistry. Sequences were analyzed using a MegaBACE 500 Sequencer (Amersham Biosciences, Buckinghamshire, England) with MegaBACE 500 Sequence Analyzer (version 2.4) software.

The GenBank/EMBL databases were used for homology searches using the BLAST program (National Center for Biotechnology Information, U.S. National Institute of Health, Bethesda, MD).

Sequence analysis

The 16S rDNA nucleotide sequences obtained in this study were aligned with a selection of 16S rDNA sequences of *Pantoea* species from GenBank with the DNAMAN software (version 5.2.9, Lynnon Biosoft, Quebec, Canada), using an optimal alignment and a dynamic method according to Feng and Doolitlle (1987). Phylogenetic trees were constructed with the neighbor joining method (Saitou and Nei, 1987) and evolutionary distances calculated according to the method of Jukes and Cantor (1969) and Kimura (1980), using the DNAMAN software package. Bootstrap analysis of the data, based on 1000 permutations was used to assess the stability of relationships.

Results

Recovery from plants

P. ananatis was not isolated from diseased plants. The lesions on onions with symptoms similar to leaf and seed stalk necrosis were caused by *Pseudomonas syringae*, which was frequently isolated from these plants on a Milk-Tween medium (Goszczynska and Serfontein, 1998) and confirmed by pathogenicity tests and LOPAT characteristics (Lelliot *et al.*, 1966).

Yellow, mucoid colonies were consistently isolated on NA from diseased onions with center rot-like symptoms. Yellow bacteria were gram-negative rods, oxidase negative, catalase positive, fermentatively utilized glucose and produced gas from glucose. Five representative strains were identified as *Enterobacter cloacae* using the Biolog system, with a similarity index of 0.82-0.85. Five strains were positive in the following tests on the API 20E strips: β-galactosidase, arginine dihydrolase, lysine decarboxylase, utilization of citrate, tryptophane deaminase, production of acetoin, and acid production from glucose, mannitol, rhamnose, sucrose, melibiose, amygdalin and arabinose. They were negative in the following tests: ornithine decarboxylase, H_2S production, urease, indole production, gelatinase, acid from inositol and sorbitol. The strains induced leaf rot on onion leaves in pathogenicity tests.

Rot of onions in South Africa and its causal agent *E. cloacae* were similar to that reported by Bishop (1990) and Schwartz and Otto (2000b) in the USA.

Recovery from seed

Yellow bacteria were recovered from most seed samples on NA. Preliminary identification using biochemical tests showed these strains were not *P. ananatis* (data not shown). All were negative in a stub inoculation pathogenicity test. On PA 20, *P. ananatis* was isolated from four of 28 *A. cepa* seed samples tested, but not from seed of *A. fistulosum* or *A. porri*. Characteristic yellow colonies, 3-4 mm in diameter, shiny, drop-shaped with small, granular, darker inclusions were visible on a PA 20 medium after 6-7 days of incubation at 25°C (Goszczynska *et al.*, 2006). The number of *P. ananatis*-like colonies recovered from the infested seed lots ranged from 2.2 x 10^3 CFU/gram to 5.0 x 10^6 CFU/gram of seed (Table 1). The strains from onion seed produced yellow colonies on NA, were gram-negative rods (1.5-2.0 µm length and 0.5-0.75 µm width), motile, oxidase negative and catalase positive. All strains fermentatively utilized glucose and produced indole from tryptophane. They did not produce gas from glucose nor hydrogen sulphide from cysteine.

One pathogenic strain per seed lot was selected for further characterization: BD 336 and PA 4 from two different experimental onion seed lots, harvested two years prior to testing and BD 390 from the seed produced by a farmer for his own use. None of the strains from the commercial seed cv. Granex 33 was pathogenic on onion cv. Granex 33 in a stub test and they were excluded from the study.

Pathogenicity test

All eight selected strains from onion were pathogenic to onion cv. Granex 33, but not to leek (Table 2). Strains BD 336, BD 310 and PA 4 did not produce disease symptoms on onion cv. Pyramid, all other strains did. Branching onion (*A. fistulosum*) cv. White Welsh was susceptible only to BD 287. All strains, including BD 287, induced identical symptoms on susceptible onion plants. Two to three days after inoculation, water-soaked spots appeared at the tips of leaves that expanded into longitudinal, bleached, green lesions with chlorotic margins. After two weeks, plants were completely wilted (Figure 1). Colonies recovered from onion plants with disease symptoms were yellow, gram-negative rods, oxidase negative and utilized glucose as a facultative anaerobe. All strains, except re-isolated BD 287 produced indole.

Two strains from *Eucalyptus*, LMG 20103 and LMG 20104, did not induce the disease on onions or leek. Control plants inoculated with water did not develop symptoms, nor were yellow bacteria isolated from leaves of these plants on NA and PA 20.

Physiological and biochemical characterization

The metabolic profiles of the eight strains from onion and two strains from *Eucalyptus* on Biolog GN microplates were most similar to the database profile of *P. agglomerans*, with a mean similarity index of 0.47. The type strains of *P. ananatis*, LMG 2665^T and *P. agglomerans*, LMG 1286^T were both identified as *P. agglomerans* by Biolog with a similarity index 0.51 (*P. ananatis* was not in the Biolog database). There was consistency in utilization of 66 substrates but some strains varied in the utilization of glycogen, adonitol, turanose, α-hydroxybutyric acid, bromosuccinic acid, succinamic acid, D-serine and uridine. The type strain of *P. agglomerans* and BD 287 gave nearly identical reactions and differed from others by not utilizing 11 substrates: Tween 40, Tween 80, gentiobiose, lactulose, D-melibiose, D-raffinose, D-sorbitol, citric acid, formic acid, D-glucosamic acid and quinic acid. Strain BD 287 did not utilize D-arabitol and psicose and LMG 1286^T did not utilize α-lactose and acetic acid.

Physiological and biochemical profiles obtained by using the API 20E and API 50CHE systems revealed that South African *P. ananatis* had similar biochemical characteristics and resembled the American and *Eucalyptus* strains and the type strain of *P. ananatis* (Table 3). In the API 20E tests, LMG 2665^T, BD 301, BD 309 and BD 315 were identical. African BD 390 and PA 4 differed by giving a positive reaction in the API 20E inositol test. Strain BD 310 did not produce β-galactosidase. The three profiles were identified by the APILAB program as *Pantoea* spp. 2. South African BD 336 differed from the others by not utilizing citrate and not producing acid from rhamnose and sucrose. However, in the API 50CHE acid from sucrose was produced after 48 hours. The profile was not recognized by the program. Unlike *P.ananatis* from onion and LMG 2665^T, two strains from *Eucalyptus* liquefied gelatin (Coutinho *et al*.,2002).

P. agglomerans LMG 1286^T and BD 287 differed from the other strains by the inability to utilize citrate, produce indole from tryptophan and acid from sorbitol.

There was consistency in the ability of the twelve strains to produce acid from 35 substrates (15 positives and 20 negatives) on the API 50CHE strips (Table 3). The type strain of *P.*

agglomerans and BD 287 differed from the other strains by not producing acid from glycerol, inositol, D-sorbitol, D-raffinose and D-arabitol.

16S rDNA sequence analysis

In this study, almost complete 16S rDNA sequences (~1500 bp) were determined for the four South African and four American strains. The sequences were deposited into the GenBank database. Accession numbers are shown in Table 2.

A BLAST search of the EMBL/GenBank database conducted with the sequences revealed a high degree of sequence identity (≥ 98%) with previously determined sequences of bacteria belonging to the genus *Pantoea*. The seven indole-producing strains had the highest homology to the 16S rDNA of *P. ananatis* strains LMG 20103 (accession number AF364847) and LMG 20106 (AF364844), ranging from 98.9% for BD 309 and BD 336 to 99.7% for BD 301 and BD 315. The strain BD 287 (Hattingh and Walters, 1981) had the highest homology (99.7%) to the 16S rDNA sequence of *P. agglomerans* strain LMG 2565 (Z96082).

Figure 2 shows the phylogenetic relationship derived from a neighbor-joining analysis of the pairwise comparison among the 16S rDNA sequences of eight strains from this study with nine sequences of four described species of the genus *Pantoea*. *Klebsiella pneumoniae* DSM 30104^T (X87276) was used as an outgroup taxon. Phylogenetic trees constructed by two different methods, with Jukes-Cantor and Kimura algorithms, were identical (not shown). Five strains, three American (BD 301, BD 310, BD 315) and two South African (BD 390, PA 4), formed a cluster with *P. ananatis* (syn. *E. uredovora)* at 72% confidence value from bootstrap analysis. The strain BD 287 grouped closely (77% confidence value) with *P. agglomerans* (syn. *E. herbicola*, *E. milletiae*). The sequences of the South African BD 336 and BD 309 from the USA clustered with *P. agglomerans* on phylogenetic trees. Some strains of *P. ananatis* from rice studied by Cother *et al*. (2004) also grouped with *P. agglomerans* on phylogenetic trees of the 16S rRNA gene sequences.

Discussion

Walcott *et al*. (2002) suggested that the center rot pathogen, *P. ananatis,* was introduced to the USA on infested onion seed lots produced in South Africa. The suggestion was based on the description of a similar disease of onion, stalk and leaf necrosis, reported in South Africa

in 1981(Hattingh and Walters, 1981). The causal agent of leaf and seed stalk necrosis, although pathogenic to onion, is not *P. ananatis*. Nutritional and genotypic characteristics of a representative strain, BD 287, closely resembled those of the *P. agglomerans* type strain, LMG 1286^T (Table 3, Figure 2). Our results are supported by those of Verdonck *et al.* (1987) and Beji *et al.* (1988) who also identified BD 287 as *P. agglomerans* in their taxonomic investigations.

In this study, *P. ananatis* pathogenic to onion was detected in three South African onion seed lots. Three strains isolated from onion seed lots induced the same symptoms on onions as those caused by strains isolated from diseased plants in the USA (Figure 1). There were, however, differences in susceptibility among onions cultivars (Table 2), providing an opportunity for the selection of tolerant material. Interestedly, *P. ananatis* causing leaf blight of *Eucalyptus* did not induce the disease on onions in pathogenicity tests done in this study, suggesting that *Eucalyptus* strains could be host specific (Azad et al., 2000).

When comparing the South African strains, the type strain *P. ananatis* and the American strains associated with center rot, by means of the Biolog system, we observed similar results in the utilization of 95 different carbon sources. With API 20E and API 50CHE strips, the profiles for the majority of onion strains were also similar to each other and to the reference strains of *P. ananatis*. Three strains (BD 336, BD 309 and BD 310) varied slightly in a few biochemical characteristics (Table 3). The production of acetoin and acid from L-rhamnose and methyl-α-D mannopyranoside differed among the 18 strains of *P. ananatis* studied by Mergaert *et al.*, 1993, while β-galactosidase and citrate utilization were reported to be positive for all strains. *P. ananatis* from *Eucalyptus* (this study, Coutinho *et al.*, 2002) and rice (Cother *et al.*, 2004), also showed a few biochemical differences from those characteristic for the species.

The genotypic characterization of the three strains from onion seed confirmed that South African strains are *P. ananatis*. Although the seed strain BD 336, and the American BD 309 clustered with *P. agglomerans* on phylogenetic trees (Figure 2), their 16S rDNA sequences were 98.8% homologous to the *P. ananatis* type strain. Additionally, all seed strains and strains from the USA were indole positive and their Biolog and API profiles were more similar to those of *P. ananatis* from *Eucalyptus*, LMG 2665^T, and those reported for 18 strains of *P. ananatis* examined by Mergaert *et al.* (1993). We concluded that the strains isolated from onion seed belong to this species.

Our results showed that *P. ananatis* pathogenic to onion is present in South African onion seed. This, however, does not support the claim that the pathogen was introduced to the USA

on infected seed lots from South Africa in 1997 (Walcott *et al*., 2002). *P. ananatis* was detected in the USA on Vidalia onions several years prior to the 1997 epidemic (Mark *et al.*, 2002). *P. ananatis* is widely distributed throughout Georgia on many types of weeds as epiphytes (Gitaitis *et al.*, 2000) and it has been reported to cause diseases on muskmelon (Bruton *et al.*, 1986), honeydew melon (Wells *et al.*,1987) and Sudangrass (Azad *et al.*, 2000) in the USA. The center rot pathogen is transmitted via tobacco thrips, *Frankliniella fusca* (Wells *et al.*, 2002; Gitaitis *et al.*, 2003). Although *P. ananatis* from sudangrass did not cause extensive disease in other plant species studied by Azad *et al*. (2000), the possibility that *P. ananatis* was transferred from another host to onions by thrips cannot be excluded.

P. ananatis was not isolated from diseased onion plants in this study. Leaf and seed stalk blights were caused by *P. syringae*, which was also present in the majority of surveyed onion seed lots (data not shown). Center rot-like symptoms observed on the water logged field in 2005 were caused by *E. cloacae*, an opportunistic pathogen and a common component of the micro flora in water, soil and on plant surfaces (Bishop, 1990; Schwartz and Otto, 2000b). Our survey of onion fields was limited to three provinces in South Africa, and we cannot conclude that the disease was not present in other areas of the country.

P. ananatis was found in three of twenty-eight locally produced *A. cepa* seed lots, and is a potential source of inoculum for the development of center rot. The diseases on sudangrass (Azad et al., 2000), *Eucalyptus* (Coutinho et al., 2002) and onions (Walcott et al., 2002) were prevalent during spring and summer, when temperatures and relative humidity were high. Onion is a winter crop in South Africa, and the arid environmental conditions could be not conductive for the disease development (Webster et al., 1983). A rapid screening technique to detect this bacterium in seed has to be evaluated, and commercially important seeds should be tested to determine their levels of infestation.

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Table 1. Recovery of *P. ananatis* from naturally infested *A. cepa* seed on PA 20 medium [a] and its pathogenicity to onion (cv. Granex 33) in a stub inoculation pathogenicity test [b].

Seed lot	Description Cultivar	*P. ananatis* CFU/gram of seed [a]	Pathogenicity to onion 20 strains per seed lot	Strains selected for characterization
1	Commercial, Granex 33	9.4×10^3	0 / 20	-
2	Experimental, Granex type	2.4×10^6	15 / 20	BD 336
3	Experimental, Granex type	2.2×10^3	9 / 20	PA 4
4	Farmer – own use, unknown	5.0×10^6	20 / 20	BD 390

[a] Characteristic yellow colonies, 3-4 mm in diameter, shiny, drop-shaped with small, granular, darker inclusions on PA 20 medium were regarded as *P. ananatis*. Colonies were counted after 7 days of incubation. Figures represent the mean of three replicates.

[b] Results were recorded after 4 days. Positive reaction: water soaked spots that expanded into longitudinal, bleached-green lesions with chlorotic margins (Goszczynska *et al*., 2006).

Table 2. Bacterial strains characterized in this study and their pathogenicity to three onion cultivars.

Strain [a]	Origin	16S rDNA GenBank accession number [c]	Pathogenicity to onion cultivars in a greenhouse test		
			A. cepa		*A. fistulosum*
			cv. Granex 33	cv. Pyramid	cv. White Welsh
	USA [b]				
BD 301	Blackshank 15, diseased onion	AY579209	+	+	-
BD 309	Horticulture Hill 24, diseased onion	AY579210	+	+	-
BD 310	Horticulture Hill 31, diseased onion	AY579211	+	-	-
BD 315	Pans 2002-2, diseased onion	AY579212	+	+	-
	South Africa				
BD 336	Onion seed	AY530794	+	-	-
BD 390	Onion seed	AY530795	+	+	-
PA 4	Onion seed	AY530796	+	-	-
BD 287 [b]	SUH 2, leaf & stalk necrosis	AY530797	+	+	+
	Reference strains				
LMG 20103	*P. ananatis*, Eucalyptus	AF364844	-	-	-
LMG 20104	*P. ananatis*, Eucalyptus	AF364845	-	-	-
LMG 1286 T	*P. agglomerans*, type strain	AJ233423	nd	nd	nd
LMG 2665 T	*P. ananatis*, type strain	Z96081	nd	nd	nd

[a] BD and PA strains: accession numbers of the Plant Pathogenic and Plant Protecting Bacteria (PPPPB) culture collection, ARC-PPRI, Pretoria, South Africa. American strains deposited by R. Walcott, Department of Plant Pathology, University of Georgia, Athens, USA. LMG strains: accession numbers of the BCCM/LMG Culture Collection, University Gent, Belgium.

[b] Isolated in 1981, Hattingh and Walters (1981).

[c] 16S rDNA sequences of reference strains obtained from the GenBank database.

nd – not done

Table 3. Physiological and biochemical characteristics of the American and South African onion strains and reference strains of *P. ananatis* and *P. agglomerans* using the API 20E and API 50CHE systems.

Characteristic [a]	*P. ananatis*		American strains				South African strains			*P. agglomerans*	
	LMG2665^T	LMG20103 LMG20104	BD 301	BD 309	BD 310	BD 315	BD336	BD390	PA4	LMG1286^T	BD 287
API 20E											
ß-galactosidase	+	+	+	+	-	+	+	+	+	+	+
Simmons citrate	+	+	+	+	+	+	-	+	+	-	-
Production of											
Indole	+	+	+	+	+	+	+	+	+	-	-
Acetoin	+	+	+	+	+	+	-	+	+	+	+
Gelatinase	-	+	-	-	-	-	-	-	-	-	-
API 50CHE											
Acid production											
Glycerol	+	+	+	+	+	+	+	+	+	-	-
L-rhamnose	+	+	+	+	+	+	-	+	+	+	+
Inositol	+	+	+	+	+	+	+	+	+	-	-
D-sorbitol	+	+	+	+	+	+	+	+	+	-	-
Methyl-α-D-mannopyranoside	+	-	+	-	+	+	-	+	+	-	-
Amygdalin	-	+	-	+	-	+	+	+	+	-	-

Characteristic [a]	*P. ananatis*		American strains				South African strains			*P. agglomerans*	
	LMG2665^T	LMG20103 LMG20104	BD 301	BD 309	BD 310	BD 315	BD336	BD390	PA4	LMG1286^T	BD 287
D-cellobiose	+	+	+	+	+	+	+	+	+	-	+
D-lactose	+	+	+	+	+	+	+	+	+	-	+
D-melibiose	+	+	+	+	+	+	+	+	+	-	+
D-raffinose	+	+	+	+	+	+	+	+	+	-	-
D-fucose	-	-	-	-	-	-	-	-	-	-	+
D-arabitol	+	+	+	+	+	+	+	+	+	-	-
Potassium gluconate	-	-	-	-	±	-	±	-	-	-	±
Aesculin hydrolysis	+	+	+	-	+	-	+	+	-	+	+

[a] API 20E tests data was recorded after 24 hours, API 50CHE data (acid production) after 48 hours. Incubation temperature was at 30^{o}C. Symbol (+) indicates positive reaction; symbol (-) negative reaction; symbol (±) uncertain.

All strains produced acid from: L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, D-mannitol, N-acetyloglucosamine, gentibiose, arbutin, salicylin, D-maltose, sucrose and D-trehalose.

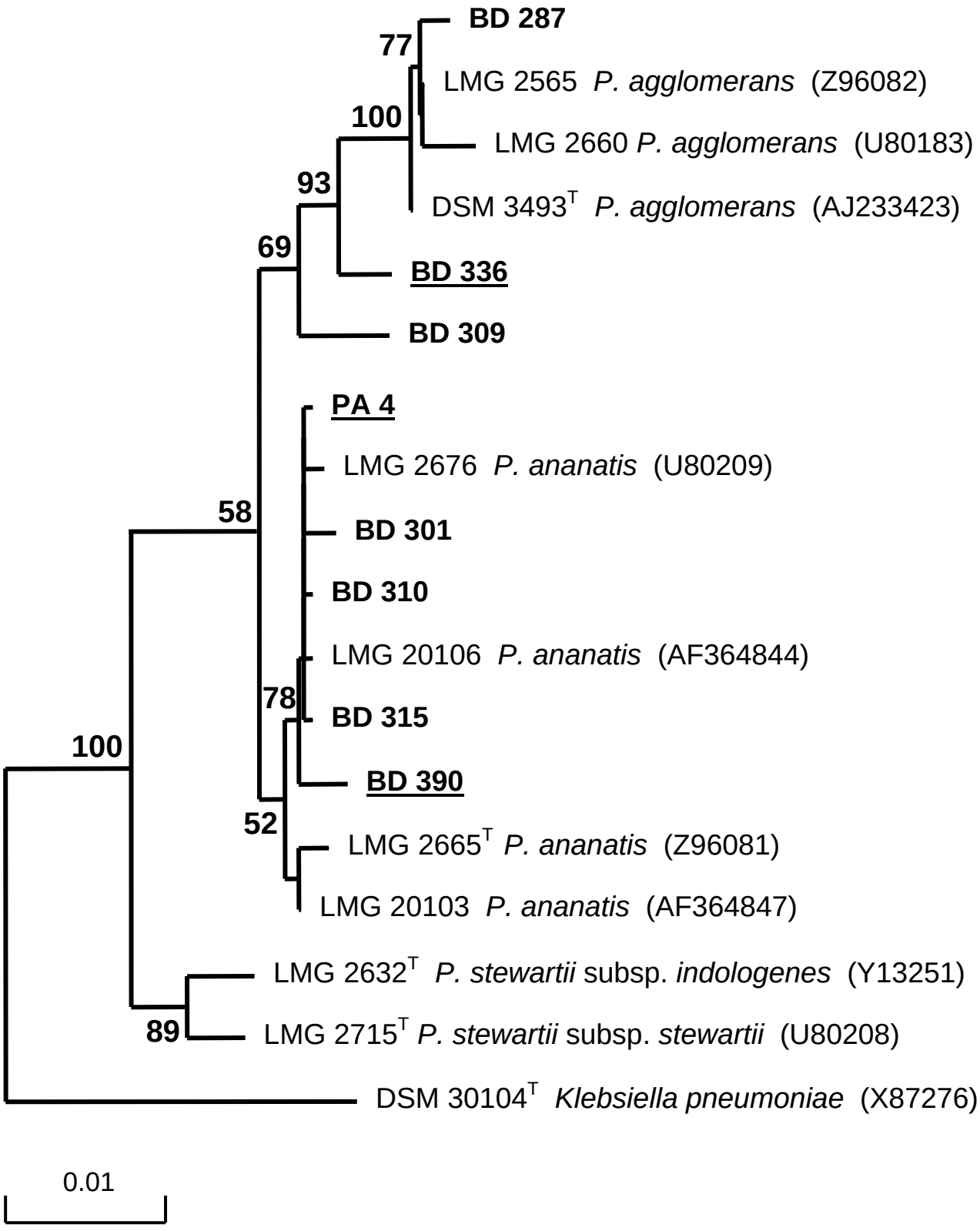
All strains were negative in the following tests: arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, H_2S, urease, tryptophane desaminase and acid production from: erythitol, D-arabinose, L-xylose, D-adonitol, methyl-B-D-xylopyranoside, L-sorbose,

dulcitol, methyl-α-D-glucopyranoside, inulin, D-melezitose, starch, glycogen, xylitol, D-turanose, D-lyxose, D-tagatose, L-fucose, L-arabitol, potassium 2-ketogluconate and potassium 5-ketogluconate.

Fig. 1. Symptoms on onion induced by the strains associated with center rot in the USA and by the South African strains isolated from onion seed.

Fig. 1

Fig. 2. Phylogenetic tree showing the relationship among selected partial 16S rDNA sequences from *Pantoea* species, including onion strains (in bold; South African strains from seed are underlined), based on pairwise comparisons using the Jukes-Cantor parameter. The sequence of *Klebsiella pneumoniae* was used as an outgroup taxon. The numbers at the nodes indicate the levels of bootstrap support based on data for 1.000 replicates; only values that are greater then 50% are shown. Accession numbers (in brackets) and the sequences of *P. ananatis*, *P. agglomerans*, *P. stewartii* and *K. pneumoniae* were obtained from the GenGank/EMBL databases.


BD 287
77
LMG 2565 P. agglomerans (Z96082)
100
LMG 2660 P. agglomerans (U80183)
93
DSM 3493T P. agglomerans (AJ233423)
69
BD 336
BD 309
PA 4
LMG 2676 P. ananatis (U80209)
58
BD 301
BD 310
LMG 20106 P. ananatis (AF364844)
78
BD 315
100
BD 390
52
LMG 2665T P. ananatis (Z96081)
LMG 20103 P. ananatis (AF364847)
LMG 2632T P. stewartii subsp. indologenes (Y13251)
89
LMG 2715T P. stewartii subsp. stewartii (U80208)
DSM 30104T Klebsiella pneumoniae (X87276)
0.01

CHAPTER 4

Isolation and Identification of the Causal Agent of Brown Stalk Rot, a New Disease of Maize in South Africa

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ABSTRACT

During 2004-2005, an unreported disease of maize (*Zea mays*) was observed on commercial fields in the Northwest and Mpumalanga Provinces of South Africa. Infected plants were stunted, with a vertical crack at the fist internode. Inside the stem, a dark brown, narrow lesion was present along the crack. Internal browning inside the stem extended upwards, reaching the top internode in some plants. Seed cobs were underdeveloped. Diseased plants were scattered in the fields and 10-70% of the crop was affected. Gram-negative, facultatively anaerobic bacteria were consistently isolated from diseased tissues. Pathogenicity tests established that representative strains induced disease symptoms similar to those observed on maize plants in the field. Physiological and biochemical characterization using the API 20E and API 50CHE systems and 16S rRNA gene sequence analyses showed that the strains belonged to the genus *Pantoea*. The results of these tests also separated the strains into two groups. The first group, giving a positive reaction in the indole test, was similar to *P. ananatis*. The second group of strains was indole negative and resembled *P. agglomerans*. The F-AFLP genomic fingerprints generated by the indole positive strains and *P. ananatis* reference strains were similar and clustered together in the dendrogram, confirming that the indole positive bacteria causing brown stalk rot on maize were *P. ananatis*. The F-AFLP fingerprints produced by the indole negative strains were distinctly different from those generated by *P. ananatis, P. agglomerans, P. dispersa, P. citrea, P. stewartii* subsp. *stewartii* and *P. stewartii* subsp. *indologenes*. The results indicated that indole negative bacteria causing brown stalk rot on maize might belong to a previously undescribed species of the genus *Pantoea*. This is the first report of a new disease on maize, brown stalk rot, caused by two bacterial species, *P. ananatis* and an undescribed *Pantoea* sp.

Additional keywords: corn, detection

Maize (*Zea mays*) is the most important grain crop in South Africa, being both the major animal feed grain and the staple food of the majority of the population. For the 2003/2004 marketing year maize was responsible for the second largest contribution to the gross value of agricultural production in the country. The South African maize industry is also the largest maize industry in Africa (39). Commercial farmers cultivate nearly three million hectares of maize per year. In the past five years, South Africa produced between 7.2 and 10.1 million tons of maize per annum, with an average of 9.2 million tons. The main maize production areas in South Africa are the Free State, Northwest and Mpumalanga Provinces. These three provinces are responsible for 85% of the total maize produced in the country (7, 39).

Numerous fungal diseases cause excessive damage to maize in South Africa (12), especially *Puccinia* common rust (13), *Cercospora* gray leaf spot (20), *Aureobasidium* leaf spot (18), *Fusarium* (14, 15, 16) and *Stenocarpella* (13) stem, cob and root rots. By contrast, only two bacterial diseases have been reported on maize in the country, leaf streak (11) caused by *Xanthomonas campestris* pv. *zeae* and bacterial stalk rot (35), caused by *Dickeya zeae*.

In January 2004, a new disease was observed in a single breeder's field in the Mpumalanga Province on an inbred female line. In December 2004 and January/February 2005 the same symptoms were also found in commercial maize fields in the Northwest and Mpumalanga Provinces. The disease was prevalent on the inbreed female and single cross female lines and a commercial hybrid SR 52. Affected plants were stunted and a vertical crack at the first internode was always present (Fig. 1A). In most diseased plants, the crack extended into the second or sometimes the third internode. When the crack was split open, a dark brown, narrow lesion was visible along the crack (Fig.1B). Internal browning inside the stem extended upwards, reaching the top internode in some plants (Fig. 1C). Seed cobs were underdeveloped. On one field, the leaf margins were soft, bleached-green and curled inwards (Fig. 1D). Diseased plants were scattered throughout the fields and 10-70% of the crop was affected.

When examined under a microscope, cut edges of symptomatic tissues consistently exhibited bacterial streaming. Gram negative bacteria producing yellow colonies were consistently isolated from diseased tissues and these were tentatively identified as belonging to the genus *Pantoea* (32). Two bacterial species of *Pantoea* have been reported to cause diseases on maize and other *Poaceae* hosts. *P. stewartii* subsp. *stewartii* causes Stewart's wilt in Europe, Asia and the Americas, but not in South Africa (8, 33). *P. ananatis* was described as an agent of leaf spot on maize in Brazil (34), stem necrosis of rice in Australia

(9) and glume and grain discoloration of rice in Japan (4). *P. ananatis* and *P. stewartii* subsp. *indologenes* were reported to cause leaf blotch on sudangrass in California (3).

The aim of this study was to identify and characterize the causal agent of brown stalk rot of maize observed in South Africa.

MATERIALS AND METHODS

Isolation of the causal agent from plants. Maize breeders and commercial farmers reported ten diseased maize fields throughout Mpumalanga and Northwest Provinces. Typical brown stalk rot symptoms were observed in these fields, on which the inbreed female, the single cross female lines and the hybrid SR 52 were cultivated. One hundred diseased plants with brown stalk rot symptoms, approximately ten plants per field, were collected in January 2004, December 2004 and February 2005.

Isolations were done from all collected plants, from at least one stalk lesion per plant and from the necrotic stripes on leaves observed in one field. Small sections of stalk or leaf tissue with symptoms were cut aseptically from the margins of lesions and macerated in 1 ml of sterile distilled water for 20 min at 25°C. Plant extracts were streaked onto nutrient agar (Difco, Sparks, MD), tryptone glucose extract agar (TGA) (Difco) and King's B (30) media. Plates were incubated at 25°C and examined after 3 to 5 days for bacterial growth. On nutrient agar, bacterial colonies were very small, approximately 1 mm in diameter. On King's B, bacterial growth was very slimy and it was difficult to distinguish single colonies. On TGA, yellow, circular, well-separated colonies, 3-4 mm in diameter, were visible 2 to 3 days after plating. After the initial isolations from approximately 20 plants, subsequent isolations were made on TGA plates only. Plates were incubated at 25°C and examined after 3 to 5 days for bacterial growth. Suspected colonies were purified by streaking onto TGA and cultures stored in milk-glycerol liquid medium (10% skim milk, 15% glycerol in distilled water) at –20°C for further analysis. The same procedure was used to re-isolate bacteria from inoculated plants.

Preliminary identification of bacteria. Ten to twenty yellow bacterial colonies per field isolated on TGA medium were purified and used in preliminary identification tests. The strains were characterized by colony morphology on TGA, motility, cell morphology, Gram stain, catalase and oxidase reaction, Hugh-Leifson oxidation/fermentation test, production of

indole, gas from glucose and hydrogen sulphide from cysteine (32), according to methods described by Fahy and Hayward (17).

Bacterial strains. Bacterial strains from maize characterized in this study are listed in Table 1. Type strains of *P. ananatis*, *P. agglomerans*, *P. dispersa*, *P. citrea*, *P. stewartii* subsp. *indologenes* and *P. stewartii* subsp. *stewartii* and seven *P. ananatis* strains isolated from sudangrass, onion and *Eucalyptus* were used as reference strains (Table 1). *P. stewartii* subsp. *stewartii* is a quarantine organism in South Africa and only DNA was used in this study. Stock cultures of all strains were maintained in milk-glycerol liquid medium at –20°C. Stock strains were transferred onto TGA plates and incubated at 25°C to recover growing cultures. Cultures were routinely checked for purity and colony characteristics on TGA.

Pathogenicity tests. Pathogenicity on maize was determined in greenhouse assays on maize cultivar SR 52, shown to be susceptible in the field. Thirteen strains from maize, two *P. ananatis* from *Eucalyptus*, five from onion and one from sudangrass were used in pathogenicity tests (Table 1). Each bacterial strain was grown on TGA plates for 24-48 hours at 25°C, and each strain was suspended in sterile distilled water to obtain approximately 10^7 CFU/ml. The concentrations were confirmed by dilution plating on TGA plates. Maize plants were inoculated immediately after preparation of the bacterial suspensions. In the first experiment, maize plants were four weeks old and in the second experiment, plants were nine weeks old. Four-week-old plants were inoculated using two methods: (a) suspensions of single strains were infiltrated into leaf tissues with a syringe and (b) suspensions of single strains were injected into stems below the first leaf whorl. Inoculated plants were placed in plastic bags and maintained in a greenhouse with natural light and 28/23°C day/night temperature. After seven days the plastic bags were removed and plants were kept in the same greenhouse. Relative humidity (RH) fluctuated between 50 and 70%. Nine-week-old plants were inoculated by injecting a few drops of bacterial suspensions into the first internode of the stem. Stem-inoculated plants were maintained in a greenhouse with natural light and at temperatures and RH described above. At least two plants were inoculated with each bacterial strain per inoculation method. Each experiment continued for ten weeks. Control plants in all experiments were inoculated with sterile distilled water. Pathogenicity tests were performed twice.

Bacteria re-isolated from symptomatic tissues were confirmed by colony morphology on TGA, Gram stain, indole production, and Huigh-Leifson and oxidase tests (10, 22).

Biochemical and physiological tests. Biochemical and physiological characteristics of the strains listed in Table 1 were examined with the API 20E and API 50CHE systems (BioMérieux, La Balme les Grottes, Montalieu Vercieu, France), according to the procedure recommended by the manufacturers. The results of the API 20E and API 50CHE tests were recorded after 24 and 48 hours of incubation at 30°C, respectively. Profiles were identified using the APILAB V4.0 identification program (BioMérieux). The data for the *P. stewartii* subsp. *stewartii* strain LMG 2715^T was obtained from the paper by Mergaert et al. (32).

The cluster analysis was performed with the combined API 20E (21 characters) and API 50CHE (49 characters) data, using the Bionumerics software (version 4.5, Applied Maths, Kortrijk, Belgium). Similarity matrixes were constructed with the Dice coefficient and cluster analyses were performed by the unweighted pair group method (UPGMA). The data was presented as an UPGMA dendrogram.

DNA extraction. Genomic DNA of bacterial strains (Table 1) was extracted by using the GenElute Bacterial Genomic DNA Kit (Sigma, Steinheim, Germany), according to the manufacturer's instructions. Purified DNA was quantified using a Dyna Quant 200 fluorometer (Hoefer, San Francisco, CA) and Hoescht H 33258 intercalating dye (Polysciences, Warrington, PA). The DNA concentration was adjusted to 25-50 ng µl $^{-1}$ with sterile water. The DNA was stored at –20°C until further analysis.

Fluorescent amplified fragment length polymorphism analysis. All strains listed in Table 1 were examined by the fluorescent amplified fragment length polymorphism (F-AFLP)-based system for the identification of plant-associated species from the genus *Pantoea* (6).

Genomic DNA, 50-100 ng from each isolate, was digested with *Eco*RI and *Mse*I (Roche, Mannheim, Germany) restriction enzymes, and then ligated to the respective adaptors for 2 hours at 20°C. Ligation reactions were diluted 1:10 in nuclease-free water for pre-amplification with Eco-00 (5'-GAC TGC GTA CCA ATT C-3') and Mse-00 (5'-GAT GAG TCC TGA CTA A-3') primers (Inquaba, Pretoria, South Africa). Each 25 µl pre-amplification reaction contained PCR buffer (10 mM Tris-HCl, pH 9.0 at 25°C; 50 mM KCl, 0.1% Triton X-100); 1.5 mM $MgCl_2$, 250 µM of each dNTPs; 100 pmol of each primer; 1 U Taq polymerase (Qiagen, Hilden, Germany) and 2 µl diluted ligation reaction. Amplification

conditions were as follows: initial denaturation at 94°C for 3 min; 20 cycles at 94°C for 30 s, 56°C for 1 min and 72°C for 1 min; and final elongation at 72°C for 5 min. Each pre-amplification reaction was diluted 1:50 in nuclease-free water prior to selective amplification with Mse-CG (5'-GAT GAG TCC TGA CTA ACG-3') and fluorescently labeled Eco-G (5'-GAC TGC GTA CCA ATT CG-3') primers (Inquaba). A volume of selective PCR reactions was 20 µl, and each contained: PCR buffer; 1.5 mM $MgCl_2$; 250 µM of each dNTPs; 0.5 pmol Eco-G primer; 2.4 pmol Mse-CG primer; 1 U Taq polymerase and 5 µl diluted pre-amplification reaction. The selective PCR amplifications included the following steps: denaturation at 94°C for 5 min; 9 cycles of denaturation at 94°C for 30 s, initial annealing at 65°C for 30s (annealing temperature was reduced by 1°C per cycle until 56°C was reached) and elongation at 72°C for 1 min; 23 cycles at 94°C for 30 s, 56°C for 30 s and 72°C for 1 min, followed by 5 min extension at 72°C. Amplifications were performed at least twice for each strain in the Hybaid Omni Gene thermocycler (Teddington, England).

The selective amplification reactions (1 µl) were mixed with an equal volume of formamide loading buffer (95% formamide, 20 mM EDTA, bromophenol blue), heated for 3 min at 90°C and then chilled on ice for 10 min. A volume of 0.5-0.8 µl of each mixture was loaded onto the gel. The AFLP products were separated in 8% Long Ranger (LI-COR Biosciences, Lincoln, NE) denaturing gels on a LI-COR IR automated sequencer. Electrophoresis was carried out for 4 hours at 1500 V in 0.8 x TBE buffer. Images were imported into BioNumerics software. Gels were standardized with a 50-700 bp sizing standard (LI-COR) and the bands between 50 and 700 bp were analyzed (6). Similarity was calculated using the Dice correlation coefficient and cluster analysis was performed by the UPGMA method. The data was presented as an UPGMA dendrogram.

Amplification and sequencing of the 16S rRNA gene. The 16S rRNA gene fragment of thirteen strains from maize (Table 1) was amplified using the universal primers fD1 (5'-AGA GTT TGA TCC TGG CTC AG-3') and rD1 (5'-AAG GAG GTG ATC CAG CCG CA-3') (41). DNA was amplified in 50 µl reaction volumes containing PCR buffer (10 mM Tris-HCl, pH 9.0 at 25°C; 50 mM KCl, 0.1% Triton X-100); 1.5 mM $MgCl_2$; 150 µM dNTPs; 1.0 µM each primer; Taq polymerase (Promega Corp., Madison, WI) 1 unit per reaction volume and 25-50 ng DNA template μl^{-1}. Amplifications were performed according to Weisburg *at al*. (41) in a Hybaid Omni Gene thermocycler.

The amplified PCR products were cut from the gel and purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany), and then cloned with the pGEM-T Easy Vector System II kit (Promega, Madison, WI) according to the manufacturer's instructions. Plasmids with inserts were purified using the QIAprep Spin kit (Qiagen). The sequencing was performed using the Dyenamic ET Dye terminator cycle sequencing kit for MegaBACE with dideoxynucletide chain-termination chemistry. Sequences were analyzed using a MegaBACE 500 Sequencer (Amersham Biosciences, Buckinghamshire, England) with MegaBACE 500 Sequence Analyzer (version 2.4) software.

The GenBank/EMBL databases were used for homology searches using the BLAST program (National Center for Biotechnology Information, U.S. National Institute of Health, Bethesda, MD).

Sequence analysis. The 16S rDNA nucleotide sequences obtained in this study were aligned with a selection of 16S rDNA sequences of *Pantoea* species from GenBank with the MAFFT software, ver. 5.743 (28). Phylogenetic trees were constructed with the neighbor joining method (36) and evolutionary distances calculated according to the method of Kimura (29), using the MEGA software package, version 3.1, Kumar, Tamura, Nei, 2004 (31). Bootstrap analysis of the data, based on 1000 permutations was used to assess the stability of relationships.

RESULTS

Isolation of the causal agent. Yellow bacterial colonies were consistently isolated from all diseased tissues on TGA medium. Colonies were circular, 2-3 mm in diameter, raised, regular and shiny. One hundred and thirty-seven colonies were purified on TGA and used in

preliminary identification tests. All strains were gram negative rods, motile, oxidase negative and catalase positive. They did not produce H_2S from cysteine and gas from glucose. All strains fermentatively utilized glucose. The majority of isolates produced indole, with the exception of all strains isolated from a single field of cv. SR 52 maize in the Northwest Province.

Pathogenicity test. When four-week-old seedlings were inoculated, no lesions or other disease symptoms developed within ten weeks on any of the maize inoculated with any of the bacterial cultures.

When bacterial suspensions were injected into the stems of nine-week-old plants, all thirteen strains from maize produced brown stalk rot symptoms within six to seven weeks (Table 1). Four to six days after inoculation, small, 2-4 mm, light brown or dark green lesions developed around the inoculation point. Drops of yellow liquid slowly oozed from the lesion for two to three days. After 6 to 7 weeks, a single, long, vertical crack appeared on the injected internode. A dark brown, narrow lesion was present in an internal stem tissue along the crack. The internal browning within the stem was also observed in upper internodes. One strain from maize, BD 561, also induced the symptoms on leaves, similar to those observed in the field.

P. ananatis from *Eucalyptus*, onion and sudangrass did not cause brown stalk rot on maize. However, four strains, LMG 20103 and LMG 20104 from *Eucalyptus* and BD 315 and PA 4 from onion produced the symptoms on maize leaves, when nine-week-old plants were stem-inoculated. Four to five weeks after injection, yellow lesions appeared on young leaves. Within a week, lesions spread along leaf veins and turned bleached-green. Tissues inside the stem appeared to be healthy.

Colonies recovered from plants with disease symptoms were yellow, gram negative rods, oxidase negative and utilized glucose as a facultative anaerobe. All strains, except re-isolated BD 500, BD 502 and BD 639 (isolated from one field in the Northwest Province) produced indole. Control plants inoculated with water did not develop symptoms, nor were yellow bacteria isolated from leaves and stems of these plants on TGA.

Physiological and biochemical characterization. The results of physiological and biochemical tests using the API 20E and API 50CHE systems separated thirteen strains from maize into two groups (Fig. 2). Ten indole positive strains had similar biochemical characteristics and resembled the type strain of *P. ananatis* LMG 2665^T and *P. ananatis*

reference strains from *Eucalyptus*, onion and sudangrass (Table 2). Unlike other *P. ananatis* used in the study and 18 strains of *P. ananatis* studied by Mergaert et al. (32), three strains from maize produced acid from D-lyxose. The profiles of all these strains were identified by APILAB program as *Pantoea* spp. 2.

Three indole negative strains from maize, BD 500, BD 502 and BD 639, were similar to *P. agglomerans* LMG 1286^T, and formed a separate group on the dendrogram (Fig. 2). The ability to produce acid from inositol, D-melibiose, starch, glycogen, D-fucose and D-arabitol, distinguished three indole negative isolates from LMG 1286^T (Table 2). None of the 21 *P. agglomerans* strains studied by Beji et al. (5), 16 by Gavini et al. (19) and 70 strains studied by Verdonck et al. (40), produced acid from glycogen or D-fucose, but some did from starch, D-melibiose and D-arabitol. The profiles of LMG 1286^T, BD 500, BD 502 and BD 639 were identified as *Pantoea* spp. 3.

The type strains of *P. citrea, P. dispersa, P. stewartii* subsp. *indologenes* and *P. stewartii* subsp. *stewartii* produced different profiles and did not cluster with maize strains on the UPGMA dendrogram constructed with the results of the API 20E and API 50CHE tests (Fig. 2).

F-AFLP analysis. Strains from maize, *P. ananatis* reference strains and five type strains of other *Pantoea* species generated complex DNA fingerprints from extracted genomic DNA (Fig. 3). The selective primers Eco-G/Mse-CG (6) yielded well-defined DNA fingerprints, with an average of 80 bands per isolate. Amplified DNA fragments ranged in size from approximately 50 bp to slightly greater than 700 bp and revealed a high degree of genetic diversity among the strains. Although significant differences were observed between the major groups, within each group of strains the fingerprints correlated.

Ten indole positive strains from maize generated fingerprints that were similar to *P. ananatis* and they were contained in a cluster with *P. ananatis* type strain, LMG 2665^T, and *P. ananatis* reference strains (Fig. 3). The similarity values among these strains were between 73 and 96%.

F-AFLP fingerprints generated by the three indole negative strains from maize were almost identical (88 to 94% similarity), but clearly different from that produced by the indole positive isolates and the type strains of *P. ananatis*, *P. agglomerans*, *P. citrea*, *P. dispersa*, *P. stewartii* subsp. *stewartii* and *P. stewartii* subsp. *indologenes.*

16S rDNA sequence analysis. The 16S rDNA sequences of thirteen strains from maize obtained in this study were deposited in the GenBank database. Accession numbers are listed in Table 1.

BLAST searches with the 1.5 kb sequences showed that 16S rDNA of ten indole positive strains had the highest homology (99.3 to 99.9%) to the 16S rDNA of *P. ananatis* strains LMG 20103 (accession number AF364847), LMG 20106 (AF364844) and PA 4 (AY530796). The 16S rDNA sequence of the indole negative strains had the highest homology, 99.6%, to *P. agglomerans* DSM 3493^T (AJ233423).

Figure 4 shows the phylogenetic relationship derived from a neighbor-joining analysis of the pairwise comparison among the 16S rDNA sequences of thirteen strains from this study with fifteen sequences of five described species of the genus *Pantoea*. *Enterobacter cloacae* ATCC 13047^T (AJ251469) *Klebsiella pneumoniae* DSM 30104^T (X87276) and *Escherichia coli* ATCC 11775^T (X80725) were used as the outgroup taxons. Phylogenetic evaluation confirmed the division of strains from maize into two groups at 99% confidence value from bootstrap analysis. The tree demonstrated clearly that the sequences of all indole positive isolates from maize clustered with *P. ananatis*. Three indole negative strains grouped closely with *P. agglomerans*. However, the three sequences formed a subcluster in the tree, separate from *P. agglomerans*, at a high, 91% confidence value.

DISCUSSION

In this study, we describe a new disease on maize, brown stalk rot, caused by two species of bacteria belonging to the genus *Pantoea*, *P. ananatis* and what appears to be a previously undescribed *Pantoea* sp. Koch's postulates were confirmed using thirteen strains, which were subjected to further taxonomic investigations.

Nutritional and biochemical characterization using the commercial systems API 20E and API 50CHE, showed that the ten strains from maize had similar biochemical characteristics and resembled the type strain of *P. ananatis* and *P. ananatis* reference strains (Fig 2, Table 2). Although all indole positive, some strains varied slightly in a few biochemical tests from that characteristic for the species (32), such as producing acid from D-lyxose but not from D-sorbitol or arbutin. Several *P. ananatis* from *Eucalyptus* (10), rice (9) and onion (22) also showed a few unusual biochemical properties. The genotypic characterization confirmed that the ten strains were *P. ananatis*. The 16S rDNA sequences of all indole positive isolates were 99.3 to 99.9% homologous to that of *P. ananatis* and all clustered with *P. ananatis* in a

neighbor-joining phylogenetic tree (Fig. 4). The AFLP genomic fingerprinting technique was demonstrated as a rapid, discriminatory method not only to determine the taxonomic diversity of bacteria (37), but also to identify them to the species level (2, 24, 25, 27). The F-AFLP fingerprints produced by the ten indole positive strains using selective primers Eco-G/Mse-CG (6) strongly resembled that yielded by *P. ananatis* (Fig. 3). We conclude that the indole positive bacteria associated with brown stalk rot of maize in South Africa are *P. ananatis*.

Nutritional and physiological characteristics of the three indole negative strains, BD 500, BD 502 and BD 639 were similar to *P. agglomerans* LMG 1286^T (Fig. 2, Table 2) and their 16S rDNA sequences were 99.6% homologous to the *P. agglomerans* type strain. However, the F-AFLP fingerprints generated by these three strains, which were almost identical to each other, were distinctly different from that of *P. agglomerans* and other *Pantoea* species used in the study. BD 500, 502 and BD 639 could also be differentiated from LMG 1286^T by giving positive reaction in the tryptophane deaminase test and not producing acid from amygdalin on the API 20E strips (Table 2). Therefore, the taxonomic status of these three strains, that were isolated from only one field of maize with brown stalk rot (Table 1), needs to be further investigated.

Two different bacterial species of the genus *Pantoea* were reported to cause similar disease symptoms on other crops. *P. ananatis* and *P. agglomerans* were isolated from onion (21, 23) and proved to induce indistinguishable symptoms on this host in pathogenicity tests (22). *P. punctata* and *P. citrea* were both isolated from mandarin orange (26). *P. ananatis* and *P. stewartii* subsp. *indologenes* were causing leaf blotch of sudangrass (3) and rot of pineapple (32, 38). *P. ananatis* and *Pantoea* spp. isolated from diseased maize produced identical brown stalk rot symptoms on maize SR 52 when the pathogens were injected into the stems of nine-weeks-old plants. However, the disease did not develop following leaf inoculation. Only one strain, *P. ananatis* BD 561, produced the leaf stripes observed in a field, but failed to induce stalk rot when the leaf was inoculated. The results suggested that brown rot develops in stalks if the pathogen enters into plants through the stem. The most damaging insect pests of maize in South Africa are several species of stalk borers (1). The larvae enter the stem by boring a hole, usually some distance above the ground and bores upward in the stem. Stalk borer was present on several maize fields with brown stalk rot and *P. ananatis* was occasionally isolated from the larvae in this study (data not shown). It is possible that stalk borers play a role in the spreading of the brown stalk rot pathogens.

The sudden appearance of brown stalk rot prompted investigations into potential sources of inoculum. A strain associated with the leaf blotch disease of sudangrass in the USA (3) was non pathogenic on maize, but two strains from onion generated leaf symptoms on this plant. Center rot disease of onion has not been found in South Africa on plants and the pathogen was only isolated from onion seed (22), eliminating that host as a source of disease on maize. Although two strains of *P. ananatis* from *Eucalyptus* used in the study did not induce stalk rot on maize, they produced lesions on leaves in pathogenicity tests when injected into the stem. Bacterial blight and dieback of *Eucalyptus* was first observed in South Africa in 1998 (10), six years before the appearance of brown stalk rot on maize. The possibility that *P. ananatis* was transmitted into maize from *Eucalyptus* by an unknown vector cannot be excluded.

The impact of brown stalk rot on maize yield and quality has not been measured. Disease was most severe on inbreed and single cross female lines. This is of considerable concern, as the lines represent some of the crucial breeding stock on which the maize industry is based. More research is required to understand the epidemiology of this new disease, and to develop the management strategies to reduce its expansion.

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Table 1. Strains of *Pantoea* characterized in this study.

Strain [a]	Host and origin MP-Mpumalanga Province NW-Northwest Province	16S rDNA (GenBank accession number) [b]	Pathogenicity test on maize [c]
Strains isolated from maize with brown stalk rot symptoms			
BD 435	Maize, stalk, MP, field 1	AY898642	Brown stalk rot
BD 442	Maize, stalk, MP, field 2	AY898643	Brown stalk rot
BD 500	Maize, stalk, NW, field 3	DQ849042	Brown stalk rot
BD 502	Maize, stalk, NW, field 3	DQ849043	Brown stalk rot
BD 639	Maize, stalk, NW, field 3	DQ512489	Brown stalk rot
BD 543	Maize, stalk, NW, field 4	DQ133545	Brown stalk rot
BD 561	Maize, leaf, NW, field 4	DQ133546	Brown stalk rot Leaf stripe
BD 577	Maize, stalk, MP, field 5	DQ133547	Brown stalk rot
BD 588	Maize, stalk, MP, field 6	DQ133548	Brown stalk rot
BD 602	Maize, stalk, MP, field 7	DQ195522	Brown stalk rot
BD 622	Maize, stalk, MP, field 8	DQ195523	Brown stalk rot
BD 640	Maize, stalk, MP, field 9	DQ195524	Brown stalk rot
BD 647	Maize, stalk, MP, field 10	DQ195525	Brown stalk rot
P. ananatis reference strains from other hosts			
BD 301	Onion, USA	AY579209	No symptoms
BD 315	Onion, USA	AY579212	Leaf stripe
BD 333	Onion, SA	DQ512490	No symptoms
BD 336	Onion, SA	AY530794	No symptoms
PA 4	Onion, SA	AY530796	Leaf stripe
0197-28	Sudangrass, USA	-	No symptoms
LMG 20103	Eucalyptus, SA	AF364847	Leaf stripe
LMG 20104	Eucalyptus, SA	AF364844	Leaf stripe
Type strains			
LMG 2665^T	*P. ananatis*	Z96081	-
LMG 1286^T	*P. agglomerans*	AJ233423	-
LMG 2603^T	*P. dispersa*	DQ504305	-
LMG 2632^T	*P. stewartii* subsp.	Y13251	-

Strain [a]	Host and origin MP-Mpumalanga Province NW-Northwest Province	16S rDNA (GenBank accession number) [b]	Pathogenicity test on maize [c]
	indologenes		
LMG 2715^T	*P. stewartii* subsp. *stewartii*	U80208	-
LMG 22049^T	*P. citrea*	-	-

[a] BD and PA strains: accession numbers of the Plant Pathogenic and Plant Protecting Bacteria (PPPPB) culture collection, ARC-PPRI, Pretoria, South Africa; BD 301 and BD 315 were deposited by R. Walcott, Department of Plant Pathology, University of Georgia, Athens. LMG strains: accession numbers of the BCCM/LMG Culture Collection, University Gent, Belgium. Strain from sudangrass: D.A. Cooksey, Department of Plant Pathology, University of California, Riverside. * *P. stewartii* subsp. *stewartii* is a quarantine organism in South Africa, and only DNA was used in this study.

[b] 16S rDNA sequences of strains from maize were obtained in this study; accession numbers and sequences of other strains were obtained from the GenBank databases.

[c] Symptoms induced on the stem-inoculated maize SR 52 within six to seven weeks, when bacterial suspensions were injected into the stems of nine-week-old plants.

- not done

Table 2. Physiological and biochemical characteristics of the strains isolated from maize with brown stalk rot, and reference strains of *P. ananatis* and *P. agglomerans* using the API 20E and API 50CHE systems.

Characteristic [a]	*P. ananatis* LMG 2665^T Type strain	*P. ananatis* from onion, *Eucalyptus* and sudangrass	BD435,442,543,561, 577,588,602,622,640, 647 from maize	*P. agglomerans* LMG 1286^T Type strain	BD 500, 502, 639 from maize
API 20E					
Citrate utilization	+	d	+	-	-
Tryptophane deaminase	-	-	-	-	+
Indole	+	+	+	-	-
Gelatinase	-	d	d	-	+
Acid from					
Inositol	+	d	d	-	+
D-sorbitol	+	+	d	-	-
L-rhamnose	+	d	+	+	+
D-sucrose	+	d	+	+	+
Amygdalin	+	+	d	+	-
API 50CHE					
Acid from					
Glycerol	+	+	+	±	±
L-rhamnose	+	d	+	+	+
Inositol	+	+	+	-	+

Characteristic [a]	*P. ananatis* LMG 2665^T Type strain	*P. ananatis* from onion, *Eucalyptus* and sudangrass	BD435,442,543,561, 577,588,602,622,640, 647 from maize	*P. agglomerans* LMG 1286^T Type strain	BD 500, 502, 639 from maize
D-sorbitol	+	+	d	-	-
Methyl-α-D-mannopyranoside	+	d	d	-	-
Amygdalin	-	d	d	-	-
Arbutin	+	+	d	+	d
D-cellobiose	+	+	+	-	d
D-maltose	+	d	d	+	+
D-lactose	+	+	+	-	-
D-melibiose	+	+	+	-	+
Sucrose	+	d	+	+	+
D-raffinose	+	d	+	-	-
Starch	-	-	-	-	d
Glycogen	-	-	-	-	d
Gentibiose	+	+	d	±	+
D-lyxose	-	-	d	-	-
D-fucose	-	-	-	-	±
D-arabitol	+	+	+	-	+
Potassium gluconate	-	d	d	-	-

Characteristic [a]	*P. ananatis* LMG 2665^T Type strain	*P. ananatis* from onion, *Eucalyptus* and sudangrass	BD435,442,543,561, 577,588,602,622,640, 647 from maize	*P. agglomerans* LMG 1286^T Type strain	BD 500, 502, 639 from maize
Aesculin hydrolysis	+	d	d	+	d

[a] API 20E test data was recorded after 24 hours, API 50CHE test data was recorded after 48 hours. Incubation temperature was at 30°C. Symbol (+) indicates positive reaction; symbol (-) negative reaction; symbol (±) weak positive reaction; symbol (d) reaction differs.

All strains gave positive reactions in the following tests: API 20E – ß-galactosidase, acetoin and acid from D-glucose, D-mannitol, D-melibiose, L-arabinose; API 50CHE – acid production from: L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, D-mannitol, N-acetyloglucosamine, salicylin and D-trehalose.

All strains were negative in the following tests: API 20E – arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, H_2S, urease; API 50 CHE – acid production from: erythitol, D-arabinose, L-xylose, D-adonitol, methyl-ß-D-xylopyranoside, L-sorbose, dulcitol, methyl-α-D-glucopyranoside, inulin, D-melezitose, xylitol, D-turanose, D-tagatose, L-fucose, L-arabitol, potassium 2-ketogluconate and potassium 5-ketogluconate.

Fig. 1. Brown stalk rot symptoms on maize observed in a field. A, vertical crack on the first internode of the stem; B, long, brown lesion along the crack; C, internal browning inside the stem; D, curling of the leaf edges.

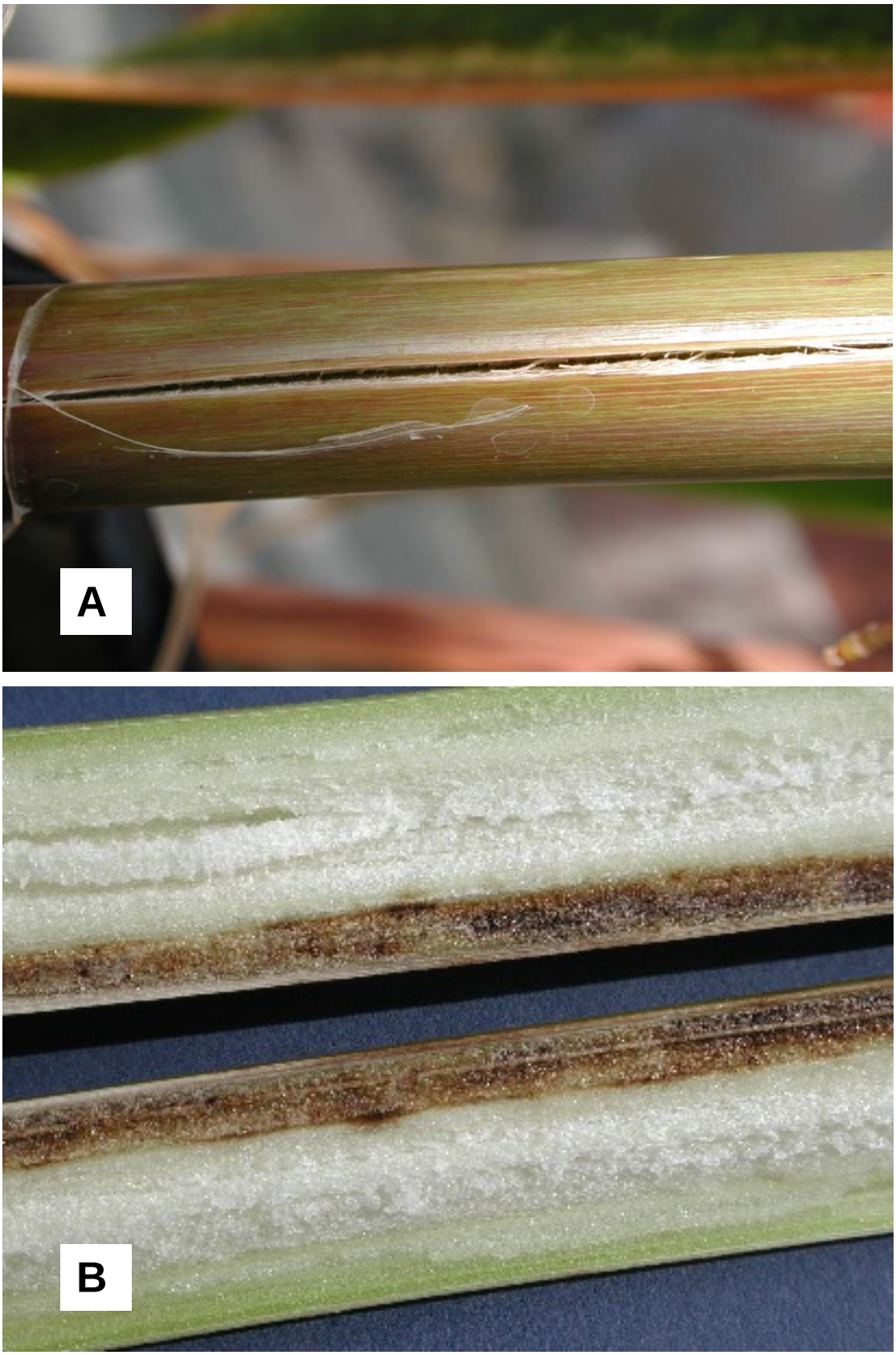


Fig. 1

Fig. 1

Fig. 2. A dendrogram based on the results of the API 20E and API 50CHE systems (BioMérieux, La Balme les Grottes, Montalieu Vercieu, France) tests, showing relationships among bacterial strains isolated from maize with brown stalk rot, *P. ananatis* reference strains, *P. agglomerans*, *P. citrea*, *P. dispersa*, *P. stewartii* subsp. *stewartii* and *P. stewartii* subsp. *indologenes.* Similarity matrixes were constructed using the Dice coefficient and cluster analyses were performed by the unweighted pair group method with the Bionumerics program (version 4.5, Applied Maths, Kortrijk, Belgium). Strains from maize are in bold (indole negative strains are underlined). The data for *the P. stewartii* subsp. *stewartii* LMG 2715^{T} was obtained from the paper by Mergaert et al. (32).

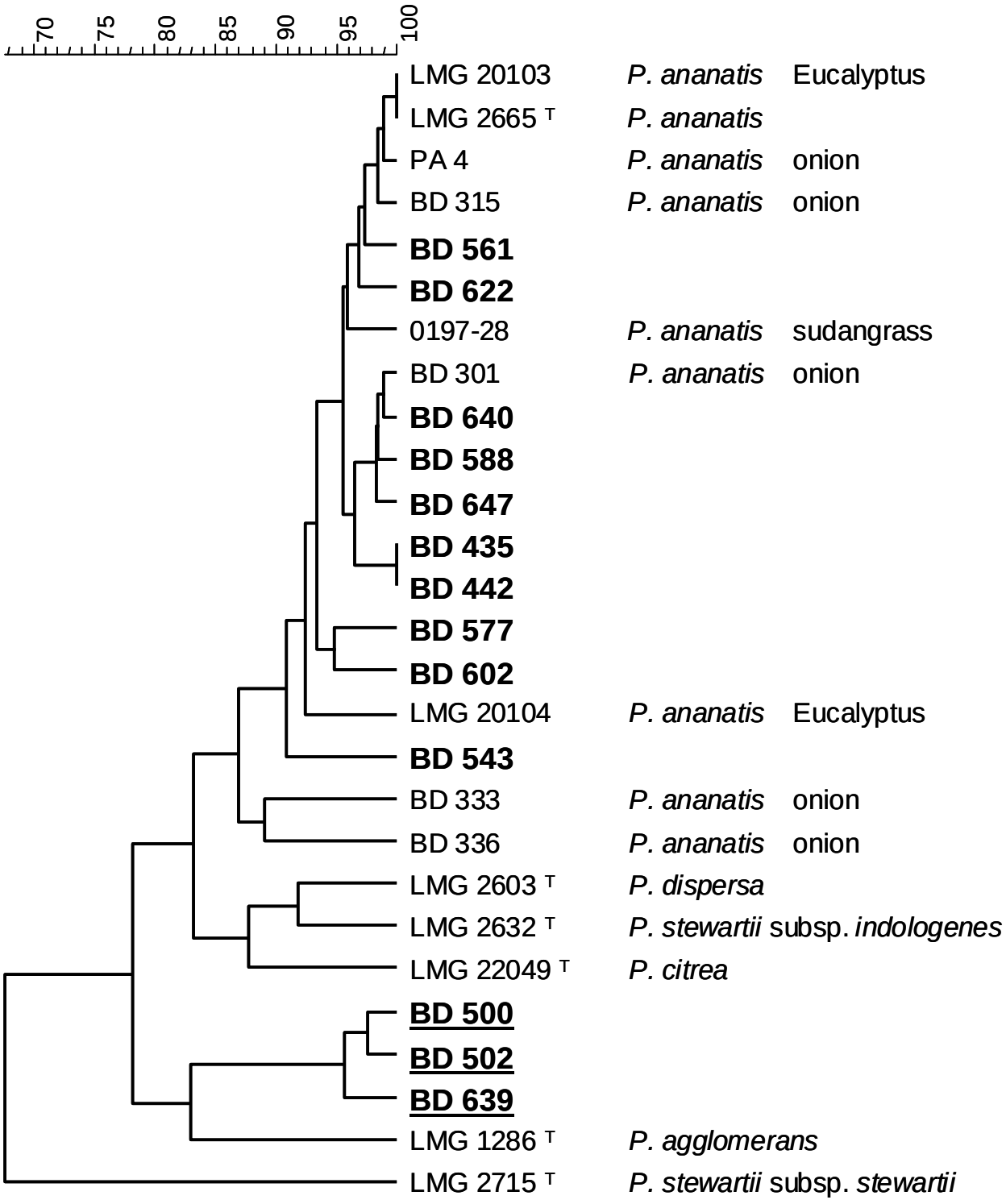

70
75
80
85
90
95
100
LMG 20103 P. ananatis Eucalyptus
LMG 2665 T P. ananatis
PA 4 P. ananatis onion
BD 315 P. ananatis onion
BD 561
BD 622
0197-28 P. ananatis sudangrass
BD 301 P. ananatis onion
BD 640
BD 588
BD 647
BD 435
BD 442
BD 577
BD 602
LMG 20104 P. ananatis Eucalyptus
BD 543
BD 333 P. ananatis onion
BD 336 P. ananatis onion
LMG 2603 T P. dispersa
LMG 2632 T P. stewartii subsp. indologenes
LMG 22049 T P. citrea
BD 500
BD 502
BD 639
LMG 1286 T P. agglomerans
LMG 2715 T P. stewartii subsp. stewartii

Fig. 3. A dendrogram showing similarity among the fluorescent amplified fragment length polymorphism (F-AFLP) DNA fingerprints of bacterial strains isolated from maize with brown stalk rot, *P. ananatis* reference strains, *P. agglomerans*, *P. citrea*, *P. dispersa*, *P. stewartii* subsp. *stewartii* and *P. stewartii* subsp. *indologenes.* The fingerprints were generated using the selective primers Eco-G/Mse-CG (6). Similarity matrixes were constructed using the Dice coefficient and cluster analyses were performed by the unweighted pair group method with the Bionumerics program (version 4.5, Applied Maths, Kortrijk, Belgium). Strains from maize are in bold (indole negative strains are underlined).

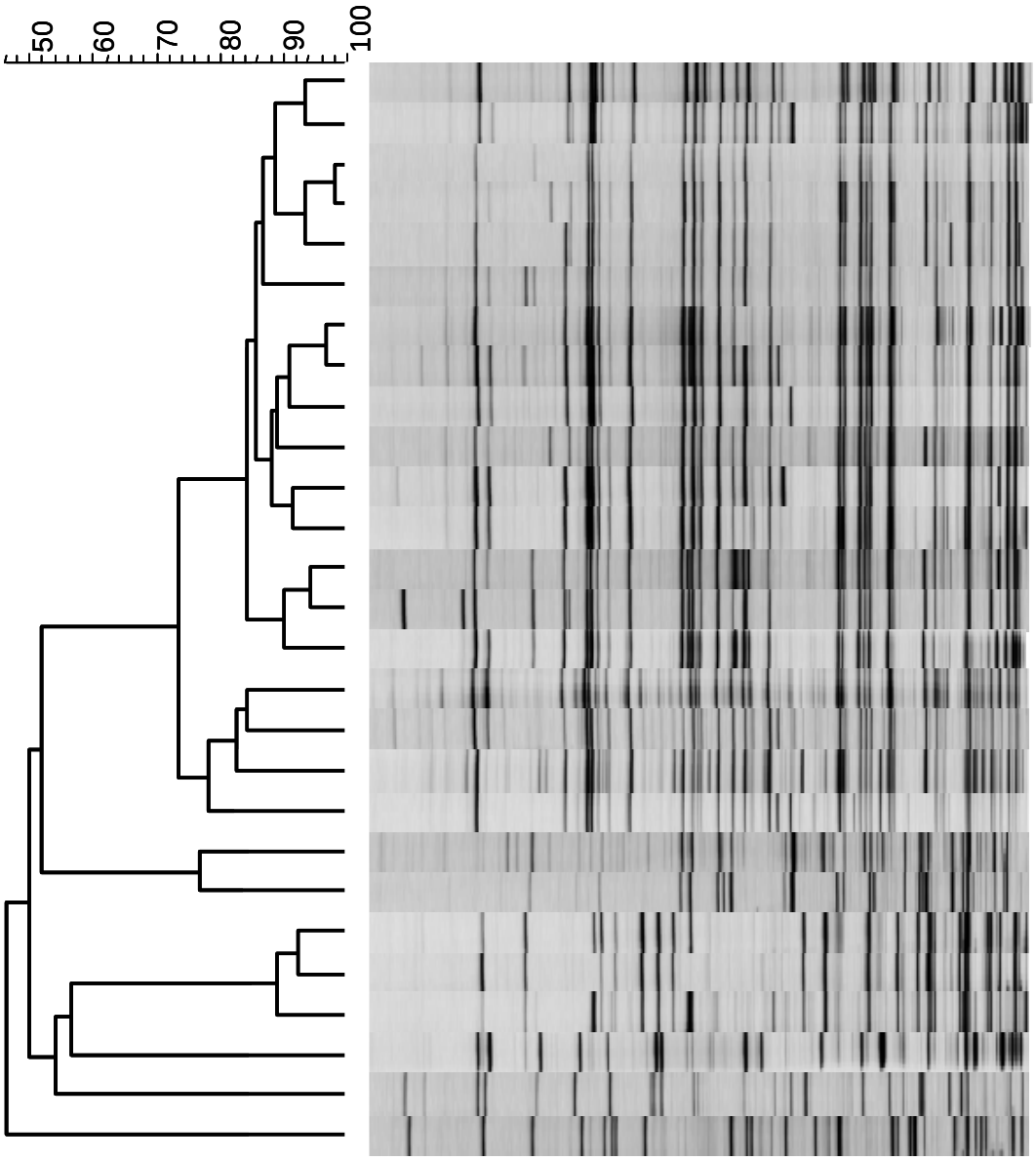
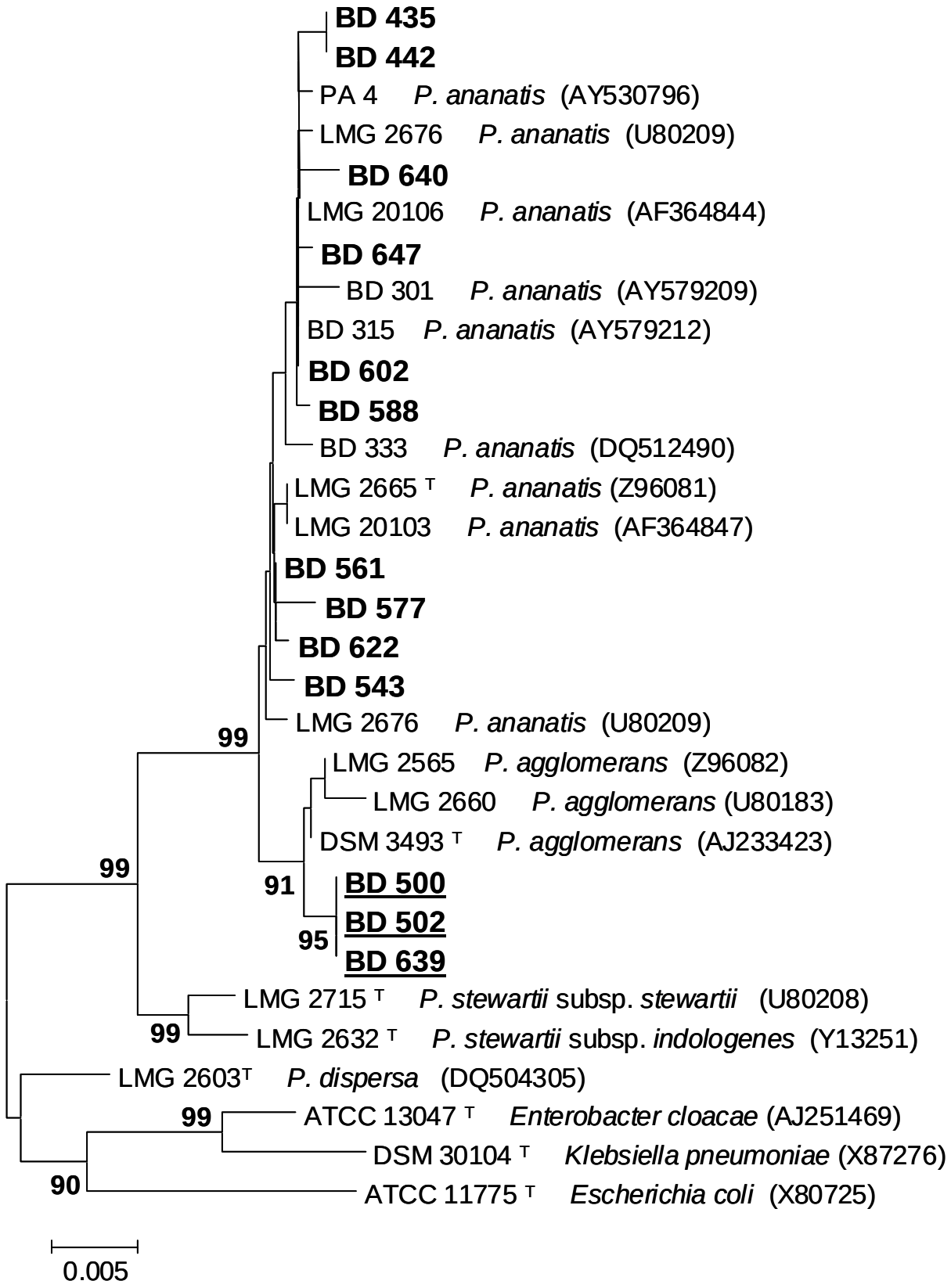

50
60
70
80
90
100
BD 640
BD 647
BD 315 P. ananatis onion
BD 435
BD 543
BD 301 P. ananatis onion
BD 561
BD 588
BD 622
BD 442
BD 577
BD 602
BD 336 P. ananatis onion
PA 4 P. ananatis onion
BD 333 P. ananatis onion
LMG 20104 P. ananatis Eucalyptus
LMG 2665 T P. ananatis
0197-28 P. ananatis sudangrass
LMG 20103 P. ananatis Eucalyptus
LMG 2632 T P. stewartii subsp. indologenes
LMG 2715 T P. stewartii subsp. stewartii
BD 500
BD 639
BD 502
LMG 1286 T P. agglomerans
LMG 2603 T P. dispersa
LMG 22049 T P. citrea

Fig. 4. Phylogenetic tree showing the relationship among selected partial 16S rDNA sequences from *Pantoea* species and strains isolated from maize with brown stalk rot (in bold, indole negative strains are underlined). Phylogenetic tree was constructed with the neighbor-joining method (39) and evolutionary distances calculated according to the method of Kimura (32), using the MEGA software package, version 3.1; Kumar, Tamura, Nei, 2004 (34). The sequences of *Enterobacter cloacae, Klebsiella pneumoniae* and *Escherichia coli* were used as outgroup taxons. The numbers at the nodes indicate the levels of bootstrap support based on data for 1.000 replicates, only values greater than 90 % are shown. Accession numbers (in brackets) and the sequences of *P. ananatis, P. agglomerans, P. dispersa, P. stewartii, E. cloacae, K. pneumoniae* and *E. coli* were obtained from the GenBank/EMBL databases. Bar represents genetic distance.


BD 435
BD 442
PA 4 P. ananatis (AY530796)
LMG 2676 P. ananatis (U80209)
BD 640
LMG 20106 P. ananatis (AF364844)
BD 647
BD 301 P. ananatis (AY579209)
BD 315 P. ananatis (AY579212)
BD 602
BD 588
BD 333 P. ananatis (DQ512490)
LMG 2665 T P. ananatis (Z96081)
LMG 20103 P. ananatis (AF364847)
BD 561
BD 577
BD 622
BD 543
LMG 2676 P. ananatis (U80209)
99
LMG 2565 P. agglomerans (Z96082)
LMG 2660 P. agglomerans (U80183)
DSM 3493 T P. agglomerans (AJ233423)
99
91
BD 500
BD 502
95
BD 639
LMG 2715 T P. stewartii subsp. stewartii (U80208)
99
LMG 2632 T P. stewartii subsp. indologenes (Y13251)
LMG 2603T P. dispersa (DQ504305)
99
ATCC 13047 T Enterobacter cloacae (AJ251469)
DSM 30104 T Klebsiella pneumoniae (X87276)
90
ATCC 11775 T Escherichia coli (X80725)
0.005