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ORIGINAL ARTICLE

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

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bacterial blight, endophyte, *Enterobacter cowanii*, *Enterobacteriaceae*, *Eucalyptus*.

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The GenBank/EMBL accession numbers for the 16S rRNA gene sequences for BCC 009 and BCC 078 are EU629163 and EU629164, respectively; and EU629165–EU629169 for the *rpoB* genes for strains BCC 008, BCC 009, BCC 011, BCC 074 and BCC 078.

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Abstract

Aims: This study was performed to identify bacterial strains isolated simultaneously with *Pantoea* species from *Eucalyptus* trees showing symptoms of bacterial blight and dieback in Uruguay.
Methods and Results: Several molecular techniques including 16S rRNA and *rpoB* gene sequencing and DNA–DNA hybridization were used to characterize the Gram-negative, facultatively anaerobic, slime-producing bacterial strains isolated along with *Pantoea* species from *Eucalyptus*. Hypersensitivity reactions (HR) and pathogenicity tests were performed on tobacco and *Eucalyptus* seedlings, respectively. The isolates clustered closely with the type strain of *Enterobacter cowanii* in both phylogenetic trees constructed. The DNA–DNA similarity between the isolates and the type strain of *Ent. cowanii* ranged from 88% to 92%. A positive HR was observed on the tobacco seedlings, but no disease symptoms were visible on the inoculated *Eucalyptus* seedlings.
Conclusions: *Enterobacter cowanii* was isolated from trees with symptoms of bacterial blight although strains of this bacterial species do not appear to be the causal agent of the disease.
Significance and Impact of the Study: This study provides the first report of *Ent. cowanii* isolated from *Eucalyptus*. Its presence in *Eucalyptus* tissue suggests that it is an endophyte in trees showing symptoms of blight.

Introduction

Eucalyptus grandis trees in Uruguay commonly exhibit symptoms of leaf blight and dieback disease. The cause of this disease is unknown, but symptoms such as water-soaked lesions with a greasy appearance are typical of bacterial infections. In 2002, symptomatic leaves and shoots were collected and isolations were made from the infected tissue. Gram-negative, facultatively anaerobic bacteria were consistently isolated from the diseased tissue. The majority of the strains was yellow pigmented and was thought to belong to *Pantoea ananatis*, the causal agent of bacterial blight and dieback on *Eucalyptus* in South Africa (Coutinho *et al.* 2002). Several nonpigmented, slime-producing strains were also isolated from the diseased material. The yellow-pigmented strains were subsequently identified as representing three novel species belonging to the genus *Pantoea* using multilocus sequence analysis (MLSA) based on *gyrB*, *rpoB*, *atpD* and *infB* gene sequences as a supporting technique (Brady *et al.* 2009). The aim of this study was to identify the nonpigmented, slime-producing bacterial strains isolated together with *Pantoea* species from *E. grandis* leaves and shoots in Uruguay. The strains were identified using 16S rRNA and *rpoB* gene-sequence comparisons as well as using DNA–DNA hybridization. In addition, pathogenicity tests were performed on *Eucalyptus* seedlings to consider their possible role in causing disease, as it was not clear whether the *Pantoea* strains or the

nonpigmented strains were responsible for the leaf- and shoot-blight symptoms observed.

Materials and methods

Bacterial strains and DNA extraction

Five nonpigmented slime-producing strains were isolated from *Eucalyptus* leaves showing typical bacterial blight symptoms including leaf spots and water-soaked lesions. The leaves were surface sterilized and crushed in sterile water, and the resulting suspension was streaked on nutrient agar and incubated at 28°C for 3 days. Single colonies were obtained by re-streaking and incubating under the same conditions. Genomic DNA was extracted from each of the bacterial strains using an alkali extraction method (Niemann *et al.* 1997) and stored at −20°C. Strains used in this study are listed in Table 1 and are maintained in a culture collection at the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria.

16S rRNA and *rpoB* gene sequencing and analysis

The almost complete 16S rRNA gene sequence was determined for two representative strains using the primers and conditions determined by Coenye *et al.* (1999). The representative strains were selected from clusters (data not shown) generated by an AFLP technique developed for the genus *Pantoea* (Brady *et al.* 2007). The *rpoB* gene sequencing was performed on all five strains according to the MLSA scheme developed for the genus *Pantoea* (Brady *et al.* 2008). The sequences were aligned using ClustalX (Thompson *et al.* 1997) and the overhangs trimmed. The Modeltest 3·7 program (Posada and Crandall 1998) was then applied to the datasets to determine the best-fit evolutionary model to apply to each gene. Maximum likelihood analysis was performed using Phyml (Guindon and Gascual 2003), by applying the models and parameters determined by Modeltest. Bootstrap analysis with 1000 replicates was performed on the trees to assess the reliability of the clusters.

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

Species	Strain	Source	Location	% DNA binding to LMG 23569^T
Ent. cowanii	LMG 23569^T = CCUG 45998^T = CIP 107300^T	Blood culture	Japan	
	BCC 008 = R-25669	*Eucalyptus*	Uruguay	
	BCC 009 = R-25670	*Eucalyptus*	Uruguay	92
	BCC 011 = R-25672	*Eucalyptus*	Uruguay	
	BCC 074 = R-21554	*Eucalyptus*	Uruguay	
	BCC 078 = R-25680	*Eucalyptus*	Uruguay	88

DNA–DNA hybridization and G+C content

High-quality DNA for DNA–DNA hybridization of strains was prepared using the method of Wilson (1987), with minor modifications (Cleenwerck *et al.* 2002). DNA–DNA hybridizations were performed using the microplate method (Ezaki *et al.* 1989) with some modifications (Cleenwerck *et al.* 2002). The hybridization temperature was 45 ± 1°C, and reciprocal reactions were performed with DNA from all strains and their variation was within the limits of this method (Goris *et al.* 1998). The values presented are based on a minimum of four replicates. *Enterobacter cowanii* type strain (LMG 23569^T) was hybridized to BCC 009 and BCC 078, while BCC 009, BCC 011 and BCC 078 were hybridized amongst each other. The G+C contents of the strains were determined by HPLC according to Mesbah *et al.* (1989).

Pathogenicity tests

Hypersensitivity reaction (HR) tests were conducted on four tobacco seedlings (*Nicotiana tabacum*) by injecting a bacterial suspension of 10^8 CFU ml^{-1} of strains BCC 008, BCC 011, BCC 074 and BCC 078 into the intercellular spaces of the leaves with a fine needle and syringe. Pathogenicity tests were performed on 12 plants of susceptible *E. grandis* × *Eucalyptus nitens* clone as previously described (Coutinho *et al.* 2002). Seedling leaves were inoculated with sterile water as a negative control and with LMG 20103 (*P. ananatis* pathogenic on *Eucalyptus*) as a positive control. The seedlings were covered with plastic bags to induce humid conditions and incubated for 2 weeks. Plants were assessed by using a 0–3 scale (0 = no disease, 3 = lesion larger than 1 cm).

Results

Sequence analyses

The 16S rRNA gene sequences of strains BCC 009 and BCC 078 were more than 99·8% similar to *Ent. cowanii* and more than 98% similar to *Enterobacter cloacae*, *Enterobacter radicincitans*, *Enterobacter asburiae* and *Enterobacter cancerogenus*. All five strains clustered closely with the type strain of *Ent. cowanii* in the *rpoB* phylogenetic tree with high bootstrap support of 100% (Fig. 1). The

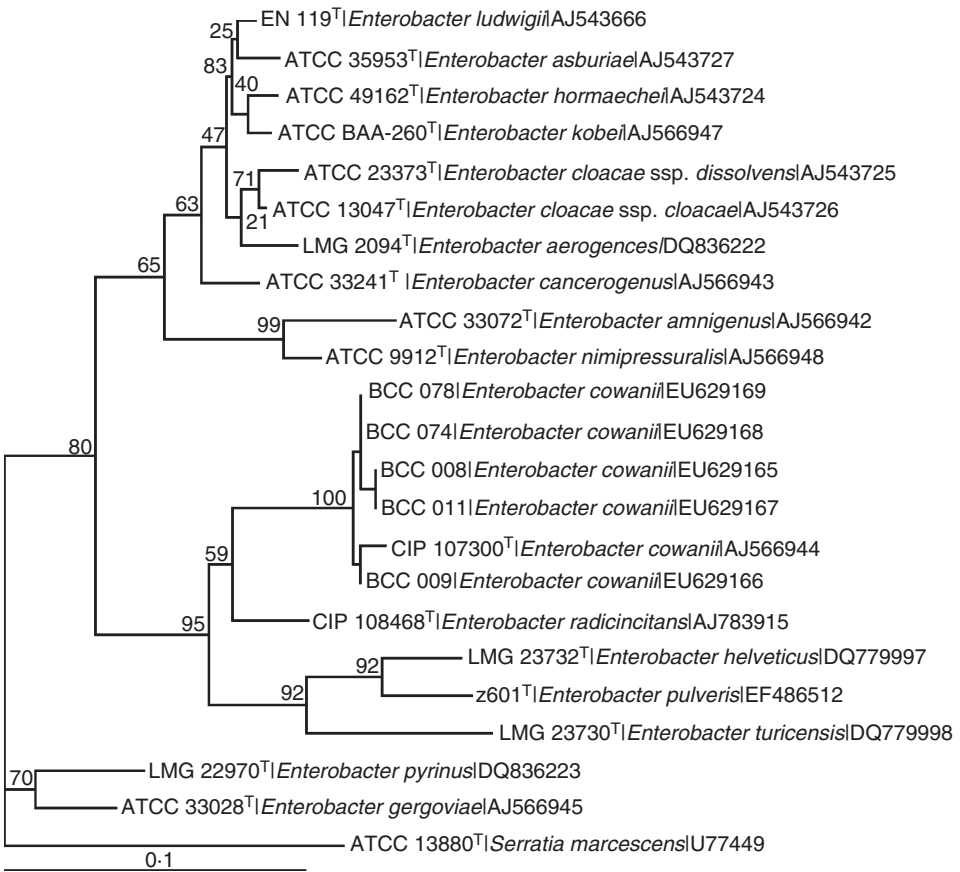

Figure 1 Maximum likelihood tree based on *rpoB* sequences of *Enterobacter* strains. Bootstrap values after 1000 replicates are expressed as percentages. *Serratia marcescens* was included as an outgroup.

topology of the *rpoB* tree was similar to that of Stephan *et al.* (2008). It was decided to hybridize the slime-producing strains to the type strain of *Ent. cowanii* based on the high 16S rRNA gene sequence similarity and the clustering of these strains in the *rpoB* phylogenetic tree.

DNA–DNA hybridization and DNA G+C content

When hybridized to BCC 009 and BCC 078, *Ent. cowanii* (LMG 23569^T) exhibited 92% and 88% DNA similarity, respectively. The DNA similarity amongst strains BCC 009, BCC 011 and BCC 078 ranged from 76% to 92%. The DNA G+C contents for strains BCC 008, BCC 009, BCC 011, BCC 074 and BCC 078 ranged from 55·8 to 56·6 mol %, which is very close to the value of 56·8 mol % reported for *Ent. cowanii* (LMG 23569^T).

Pathogenicity tests

A positive HR was observed on the tobacco seedlings inoculated with strains BCC 008, BCC 011, BCC 074 and BCC 078, which was demonstrated by the complete collapse of the leaf tissue after 24 h. The inoculated *Eucalyptus* seedlings and the negative water control displayed no symptoms during the 2 weeks in which the leaves were examined (score obtained = 0). In contrast, the leaves inoculated with *P. ananatis* developed necrotic lesions within 5 days of inoculation (score obtained = 3).

Discussion

Results of this study demonstrated that the nonpigmented, slime-producing bacterial strains isolated from the internal parts of *Eucalyptus* tissue together with *Pantoea* species belong to *Ent. cowanii*. This identification was clear from the phylogenetic tree based on *rpoB* gene sequence comparisons, which was strongly supported by high bootstrap values. The sequencing results were confirmed by DNA–DNA hybridization data and DNA G+C content of the strains. The DNA–DNA similarity values of BCC 009 and BCC 078 with LMG 23569^T, the type strain of *Ent. cowanii* and between strains BCC 009, BCC

011 and BCC 078, are both well above the recommended species definition cut-off point of 70% (Wayne *et al.* 1987). Additionally, the DNA G+C contents of the strains are within the 5 mol % difference range for species delineation (Rosselló-Mora and Amann 2001).

Species belonging to the genus *Enterobacter* are typically associated with the natural environment and are found in water, sewage and soil. Some cause diseases of trees, while others are opportunistic human pathogens (Grimont and Grimont 2006). This is the first report of the occurrence of *Ent. cowanii* on *Eucalyptus*, where it evidently can live internally in leaf tissue. Originally, *Ent. cowanii* was described for a group of clinical strains, previously identified as *Enterobacter agglomerans* (reclassified as *Pantoea agglomerans*) in routine diagnostic laboratories (Inoue *et al.* 2000). Only three of the 16 recognized species belonging to *Enterobacter* have previously been isolated from diseased trees, i.e. *Ent. cancerogenus*, *Enterobacter nimipressuralis* and *Enterobacter pyrinus* (Grimont and Grimont 2005a). In this regard, the occurrence of the bacterium on *Eucalyptus* is perhaps not unusual.

Pathogenicity tests indicated that *Ent. cowanii* strains probably do not play a primary role in the bacterial blight and dieback of *Eucalyptus* in Uruguay. The positive results of the HRs on tobacco seedlings indicate that *Ent. cowanii* can perhaps contribute to disease under environmental conditions favourable to the bacterium. However, it is most likely that *Ent. cowanii* strains isolated in this study are endophytic and were coincidentally isolated together with *Pantoea* species, which may cause the leaf symptoms observed.

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

This study concerns the first report of *Ent. cowanii* isolated from diseased *Eucalyptus*. Although the bacterium is most probably not the primary pathogen of bacterial blight and dieback in Uruguay, its occurrence in *Eucalyptus* tissue is intriguing as its ultimate role is currently unknown. Further research on the presence and role of these bacteria in *Eucalyptus* elsewhere in South America, as well as other parts of the world, may contribute to the understanding of the ecological role of these microorganisms.

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