A FAFLP system for the improved identification of plant-pathogenic and plant-associated species of the genus *Pantoea*

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Abstract

The majority of *Pantoea* species are either plant-pathogenic or plant-associated and cause a wide variety of symptoms on a range of hosts. Identification of *Pantoea* species is difficult due to minor differences in phenotypic characteristics between them and related *Enterobacteriaceae*. Fluorescent amplified fragment length polymorphism (FAFLP) analysis was investigated for use as a rapid, molecular-based identification technique to the species level of the genus *Pantoea*. Following analysis of the band patterns generated by FAFLP, seven distinct clusters were observed, one for each validly published species of the genus. FAFLP has proven to be a rapid, reproducible identification technique for all species of the genus *Pantoea*.

Article Outline

Introduction

Identification of plant-pathogenic *Pantoea* species is difficult, due to the high degree of phenotypic similarity between species of this genus and related *Enterobacteriaceae* [20]. *Pantoea* species are typically characterised based on colony morphology, physiological and biochemical tests, and in some cases, fatty acid analysis or quinone composition. This approach has proven to be unreliable though, as identification based solely on phenotypic characteristics has led to the misidentification of many strains belonging to the now obsolete "*Erwinia herbicola–Enterobacter agglomerans*" complex.

The genus *Pantoea* currently includes seven species, the majority of which are either plant-pathogenic or plant-associated. The most notorious of the plant-pathogenic species is undoubtedly *Pantoea stewartii* subsp. *stewartii*, the causal agent of Stewart's vascular wilt of sweet corn and maize [18], whereas *P. stewartii* subsp. *indologenes* is associated with sudangrass [3] and is thought to cause leaf spot on foxtail millet and pearl millet and rot of pineapple. *Pantoea agglomerans* is best known for causing crown and root gall of *Gypsophila paniculata* [6] and *Pantoea ananatis* causes a range of diseases on a wide variety of agricultural crops, the most recent including rice [8], *Eucalyptus* [9], maize [14] and sudangrass [3]. *Pantoea citrea* is associated with citrus fruit and causes pink disease of pineapple [11] and [15]. Although *Pantoea dispersa*, *Pantoea punctata* and *Pantoea terrea* have been isolated from the environment, they have not been found to be associated with diseases of plants. By utilising typical identification techniques such as Gram staining, oxidation/fermentation tests and the API 20E system (bioMérieux, Marcy

l'Etoile, France), strains may be correctly identified as belonging to the genus *Pantoea* but often cannot be accurately assigned to a species [10]. There are a few PCR-based techniques for the identification of *P. stewartii* subsp. *stewartii* [7] and [23] and *P. ananatis* [22], but these PCR assays cannot be applied to all seven species of the genus *Pantoea*. Sequencing of the 16S rRNA gene is also routinely used in the identification of bacterial species and is considered to be a standard element for the description of bacterial taxa. However, 16S rRNA sequences are too highly conserved to differentiate reliably between closely related species [17], as is the case for *Pantoea* (unpublished results).

Alternatively, amplified fragment length polymorphism (AFLP) analysis can be used for identification and typing purposes as it can discriminate to, or below, the species level [16]. Also, AFLP has been used extensively in the identification and classification of bacteria belonging to the *Enterobacteriaceae*, including *Escherichia* and *Erwinia* [1] and [2]. Therefore, the purpose of this study was to investigate the use of fluorescent AFLP (FAFLP) analysis [21] for identification, to the species level, of strains belonging to the genus *Pantoea*.

Bacterial strains

Thirty-five *Pantoea* strains (Supplementary Table S1), including the type strains of all seven species, and 10 type strains of plant-pathogenic species belonging to *Erwinia*, *Pectobacterium* and *Brenneria* were received from the BCCM/LMG Bacteria Collection (http://bccm.belspo.be). Fourteen additional *Pantoea* strains were obtained from culture collections and institutions in Australia, Japan, Sweden, Israel and the USA and they were deposited in the BCC Collection at the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria.

FAFLP analysis

Genomic DNA was extracted using a DNeasy™ tissue kit (Qiagen, Hilden, Germany) and stored at −20 °C. The FAFLP method of Vos et al. [21] was followed as described, but with some modifications. Between 100 and 150 ng of DNA from each of the strains was digested with 12 U *Eco*RI (Roche, Basel, Switzerland) and 8 U *Mse*I (Roche) in 5×

restriction/ligation buffer (50 mM Tris HAc, 50 mM MgAc, 250 mM KAc and 25 mM DTT). The digestion reaction was incubated at 37 °C for 2 h and then heated at 70 °C for 15 min. Double-stranded adaptors, 5 pmol *Eco*RI and 50 pmol *Mse*I, were added to the 15 µl digestion mixture, together with 5× restriction/ligation buffer, 0.3 mM ATP and 1 U T4 DNA ligase (Roche). The ligation reaction was incubated at room temperature for 2 h and then diluted 1:10 with nuclease-free water. Pre-amplification reactions were performed and contained 1× reaction buffer, 1.5 mM $MgCl_2$, 250 µM dNTPs, 100 pmol each of Eco-00 (GACTGCGTACCAATTC) and Mse-00 primer (GATGAGTCCTGACTAA), 1 U *Taq* DNA polymerase (JMR Holdings, Kent, UK) and 2 µl diluted ligation reaction product. The amplification conditions included denaturation at 94 °C for 3 min, 20 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 1 min and elongation at 72 °C for 1 min, and extension at 72 °C for a further 5 min. Following pre-amplification, each reaction was diluted 1:50 with nuclease-free water. The selective amplification reactions, in a total volume of 20 µl, contained 1× reaction buffer, 1.5 mM $MgCl_2$, 250 µM dNTPs, 0.5 pmol fluorescently labelled Eco-C primer (GACTGCGTACCAATTCC), 2.4 pmol Mse-GC primer (GATGAGTCCTGAGTAAGC), 1 U *Taq* DNA polymerase (Southern Cross Biotechnologies) and 5 µl diluted pre-amplification reaction product. The selective PCR conditions included denaturation at 94 °C for 5 min, nine cycles of denaturation at 94 °C for 30 s, annealing at 65 °C for 30 s and elongation at 72 °C for 1 min, where the annealing temperature decreases by 1 °C/cycle until 56 °C is reached. This was followed by 23 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and elongation at 72 °C for 1 min, and a further 5 min of extension at 72 °C. The separation of FAFLP fragments was performed using a LI-COR IR2 automated sequencer (LI-COR Biosciences, Lincoln, Nebraska, USA) following the protocol of Myburg et al. [13].

Gel analysis

The band patterns from the gels were analysed using GelCompar II (Applied Maths, Sint-Martens-Latem, Belgium). Gels were normalised by aligning the 700 bp sizing standards included in each gel, and the area between 50 and 700 bp, which is normalised best by the GelCompar analysis software, was selected for numerical analysis. Following

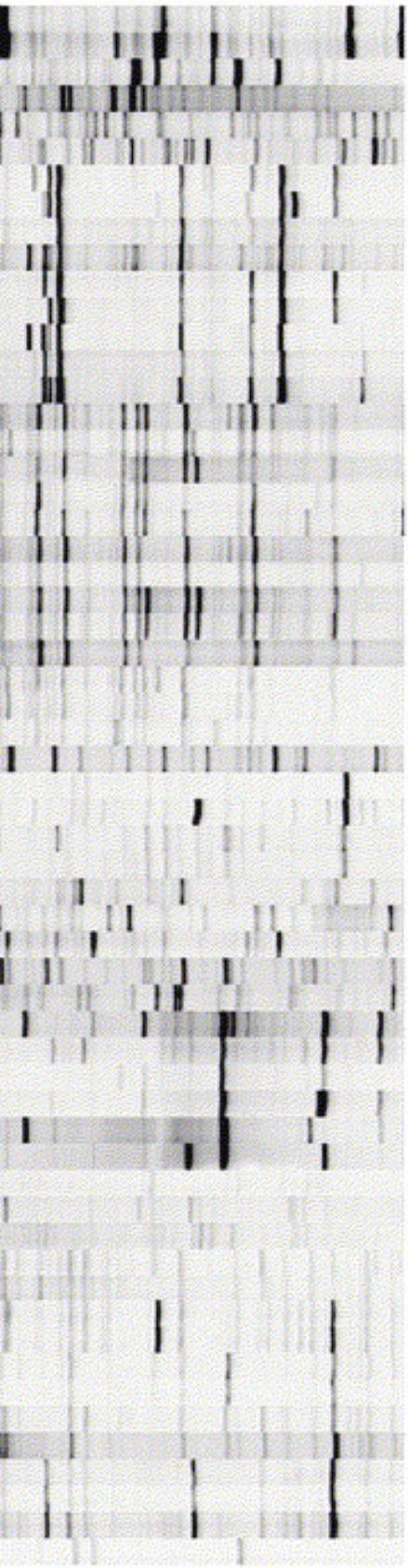
analysis, a UPGMA (Unweighted Pair Group Method with Arithmetic mean) dendrogram was constructed using the Dice similarity coefficient. An optimisation setting of 0.05% and a tolerance setting of 0.1% were applied to the analysis.

Reproducibility

Factors including DNA extraction method, DNA concentration, PCR efficiency and LI-COR gel running conditions were kept standard for all strains used in this AFLP study, in order to maintain good reproducibility. Additionally, the type strain of *P. ananatis* was included in each template preparation and separated on each LI-COR gel as a positive control. Only gels where the band pattern of the type strain of *P. ananatis* was more than 75% similar to the original band pattern were analysed and included in the results.

FAFLP analysis of *Pantoea* species

In the UPGMA dendrogram (Fig. 1), produced from FAFLP analysis of the *Pantoea* strains, seven distinct clusters, one for each species of *Pantoea*, were identified. The clusters are defined by the grouping together of banding patterns that showed a linkage level of 50% to other clusters. This level of cluster delineation was comparable to those used in previous studies [16].



LMG 23359	*Pantoea citrea*	Cluster I
LMG 23360	*Pantoea citrea*	
LMG 22049$^{T r}$	***Pantoea citrea***	
LMG 17936^{T}	*Pectobacterium cacticida*	
LMG 2386^{T}	*Pectobacterium atrosepticum*	
LMG 2404^{T}	*Pectobacterium carotovorum* ssp. *carotovorum*	
LMG 2565^{r}	*Pantoea agglomerans*	Cluster II
LMG 2660^{r}	*Pantoea agglomerans*	
LMG 1286$^{T r}$	***Pantoea agglomerans***	
LMG 2596	*Pantoea agglomerans*	
LMG 2554	*Pantoea agglomerans*	
LMG 2941	*Pantoea agglomerans*	
BCC 734	*Pantoea agglomerans*	
LMG 2572	*Pantoea agglomerans* pv. *betae*	
LMG 2553	*Pantoea agglomerans* pv. *gypsophilae*	
BCC 371	*Pantoea ananatis*	Cluster III
BCC 374	*Pantoea ananatis*	
LMG 2666	*Pantoea ananatis*	
LMG 20103^{r}	*Pantoea ananatis*	
LMG 2665$^{T r}$	***Pantoea ananatis***	
BCC 146	*Pantoea ananatis*	
LMG 20104^{r}	*Pantoea ananatis*	
BCC 112	*Pantoea ananatis*	
BCC 114	*Pantoea ananatis*	
BCC 150	*Pantoea ananatis*	
BCC 083	*Pantoea ananatis*	
BCC 098	*Pantoea ananatis*	
LMG 2676^{r}	*Pantoea ananatis*	
LMG 2709^{T}	*Brenneria rubrifaciens*	Cluster IV
BCC 746	*Pantoea punctata*	
LMG 22097^{r}	*Pantoea punctata*	
LMG 22050$^{T r}$	***Pantoea punctata***	
LMG 22098^{r}	*Pantoea punctata*	
LMG 2688^{T}	*Erwinia rhapontici*	
LMG 7034	*Erwinia psidii*	
LMG 11254^{T}	*Erwinia persicina*	
LMG 2694^{T}	*Brenneria nigrifluens*	Cluster V
LMG 2698^{T}	*Brenneria salicis*	
LMG 2603$^{T r}$	***Pantoea dispersa***	
LMG 2749	*Pantoea dispersa*	
LMG 2602^{r}	*Pantoea dispersa*	
LMG 2604^{r}	*Pantoea dispersa*	
LMG 2601^{r}	*Pantoea dispersa*	
LMG 2605	*Pantoea dispersa*	
BCC 748^{r}	*Pantoea terrea*	Cluster VI
LMG 22051$^{T r}$	***Pantoea terrea***	
BCC 747^{r}	*Pantoea terrea*	Cluster VII
BCC 103	*Pantoea stewartii* ssp. *indologenes*	
LMG 2671	*Pantoea stewartii* ssp. *indologenes*	
LMG 2673^{r}	*Pantoea stewartii* ssp. *indologenes*	
LMG 2674	*Pantoea stewartii* ssp. *indologenes*	
LMG 2630^{r}	*Pantoea stewartii* ssp. *indologenes*	
LMG 2631	*Pantoea stewartii* ssp. *indologenes*	
LMG 2632$^{T r}$	***Pantoea stewartii* ssp. *indologenes***	
BCC 118	*Pantoea stewartii* ssp. *indologenes*	
LMG 2713^{r}	*Pantoea stewartii* ssp. *stewartii*	
LMG 2715$^{T r}$	***Pantoea stewartii* ssp. *stewartii***	
LMG 2718^{r}	*Pantoea stewartii* ssp. *stewartii*	
LMG 2708^{T}	*Erwinia mallotivora*	

Fig. 1. UPGMA dendrogram based on FAFLP analysis of *Pantoea* species and plant-pathogenic *Enterobacteriaceae* isolates using the selective primer combination Eco-C/Mse-GC. The levels of similarity representing the Dice similarity coefficient are expressed as percentages. The banding patterns adjacent to each branch are normalised and background-subtracted digitised gel strips processed in GelCompar. *Pantoea* type strains are in bold. T=type strain, r=*Pantoea* reference strain, LMG=Culture Collection, Laboratorium voor Microbiologie, Ghent, Belgium, BCC=Bacterial Culture Collection, Forestry and Agricultural Biotechnology Institute, Pretoria, South Africa.

Twenty-four *Pantoea* strains used in this study were previously used in DNA hybridisation studies [4], [5], [9], [10], [11] and [12] and are regarded as reference strains (Fig. 1). Many of these strains were also investigated by protein profiling and 16S rRNA sequencing. Each of these strains fell within their intended species cluster in the AFLP dendrogram, thereby proving the usefulness of this technique for identification of *Pantoea* strains to the species level. Twenty-two of the non-reference strains used in this study clustered with their intended species, while two strains, BCC 103 (from sudangrass) and BCC 118 (from *Eucalyptus* infected with *Colletogloeopsis zuluense*), did not. Both strains were received as *P. ananatis*, but they clustered with *P. stewartii* subsp. *indologenes*. BCC 103 was isolated from sudangrass showing symptoms of leaf blotch disease, where the two causal agents were identified as *P. ananatis* and *P. stewartii* following physiological and biochemical tests [3]. BCC 103 fell into cluster VII with *P. stewartii* subsp. *indologenes*, indicating that this strain was initially misidentified as there is very little phenotypic difference between *P. ananatis* and *P. stewartii* subsp. *indologenes*. BCC 118, isolated from *Eucalyptus* showing symptoms of Colletogloeopsis canker, was thought to be one of several *P. ananatis* strains, which can exist in a synergistic relationship with the fungus *C. zuluense* [19]. BCC 112 and BCC 114 were also isolated along with *C. zuluense* and both of these strains fell into cluster III with reference strains of *P. ananatis*. However, as BCC 118 clustered with strains of *P. stewartii* subsp. *indologenes* with a 50% similarity, it is certain that this strain was originally misidentified as *P. ananatis*. This correlates with previous findings based on 16S rRNA sequencing by van Zyl [19], which indicated the possible association of two

Pantoea species with the fungal pathogen, *P. ananatis* and *P. stewartii*. In both of the above cases, strains isolated from sudangrass and *Eucalyptus* infected with *C. zuluense* could only be identified to the species level as *P. stewartii* by phenotypic tests or 16S rRNA sequencing. FAFLP analysis places both of these strains in the subspecies *indologenes*, demonstrating the ability of this technique to discriminate below the species level.

Since the original appearance of *P. ananatis* on *Eucalyptus* in 1998 [9], there have been numerous outbreaks of bacterial blight in South Africa. The FAFLP technique developed in this study is used as a routine diagnostic service by the FABI at the University of Pretoria to identify *Pantoea* strains causing the bacterial blight outbreaks. A continuously updated FAFLP database of *Pantoea* banding patterns now exists, to which possible *Pantoea* strains can be compared. This in-house database will assist us in the reliable identification of all *Pantoea* species, especially strains of the three most phytopathologically important species, *P. stewartii* subsp. *stewartii*, *P. agglomerans* and *P. ananatis*.

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Appendix A. Supplemental material

 (109 K)

f.doc Help

Microsoft Word file 1.

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