Analysis of the Phylogenetic Relationships of Strains of *Burkholderia solanacearum*, *Pseudomonas syzygii*, and the Blood Disease Bacterium of Banana Based on 16S rRNA Gene Sequences

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We determined nearly complete 16S rRNA gene sequences for 19 isolates of *Burkholderia solanacearum*, three isolates of the blood disease bacterium of bananas, and two isolates of *Pseudomonas syzygii*, the cause of Sumatra disease of cloves. The dendrogram produced by comparing all of these sequences revealed that there were two divisions, which corresponded to the results obtained previously in a restriction fragment length polymorphism analysis (D. Cook, E. Barlow, and L. Sequeira, Mol. Plant Microbe Interact. 2:113–121, 1989) and a total 16S ribosomal DNA (rDNA) sequence analysis of four isolates representing four biovars of *B. solanacearum* (X. Li, M. Dorsch, T. Del Dot, L. I. Sly, E. Stackebrandt, and A. C. Hayward, J. Appl. Bacteriol. 74:324–329, 1993). Division 1 comprised biovars 3, 4, and 5 and an aberrant biovar 2 isolate (strain ACH0732), and division 2 included biovars 1, 2, and N2, the blood disease bacterium, and *P. syzygii*. Specific nucleotides at positions 458 to 460 (UUC) and 474 (A) characterized division 2, whereas in division 1 the nucleotides at these positions were ACU and U, respectively. However, strain ACH0732 had a U at position 458, as did division 2 isolates, and G instead of U at position 474. Division 2 consisted of two subdivisions; one subdivision contained two *B. solanacearum* isolates that originated from Indonesia, *P. syzygii* strains, and blood disease bacterium strains, and the other subdivision contained all of the other division 2 isolates. Within division 1, the level of 16S rDNA sequence similarity ranged from 99.8 to 100%, and within division 2, the levels of 16S rDNA sequence similarity ranged from 99.1 to 100%. The division 1 isolates exhibited an average level of 16S rDNA sequence similarity to division 2 isolates of 99.3% (range, 99.1 to 99.5%). The occurrence of consistent polymorphisms in the 16S rDNA sequences of *B. solanacearum* strains, in particular unique 16S rDNA sequence differences in aberrant biovar 2 isolate ACH0732, and the occurrence of the Indonesian subdivision of division 2 suggest that this group is a rapidly evolving (tachtyelic) group.

The use of rRNA sequences for classification and identification of microorganisms is now routine, and analysis of 16S rRNA gene sequences provides a powerful tool for determining the phylogenetic and evolutionary relationships of microorganisms (34). The rRNA genes are useful molecular chronometers not only because of their conserved nature, high information content, and universal distribution, but also because of the rapidly expanding 16S rRNA gene sequence database that can be used for phylogenetic comparisons.

*Burkholderia solanacearum* causes bacterial wilt of a wide range of crops and is one of the most important disease bacteria in tropical and subtropical regions and some of the warm temperate regions of the world (12). *B. solanacearum* belongs to pseudomonad rRNA homology group II as determined by rRNA-DNA homology (24) and to the beta subclass of the Proteobacteria (8, 18).

*B. solanacearum* is a heterogeneous species which exhibits significant phenotypic diversity (3, 12). Traditionally, *B. solanacearum* strains have been divided into five races on the basis of host range and into five biovars on the basis of biochemical properties (10, 12). Two clusters of *B. solanacearum* strains have been identified on the basis of restriction fragment length polymorphisms (3, 4) and on the basis of the 16S ribosomal DNA (rDNA) sequences of four isolates representing biovars 1, 2, 3, and 4 (18).

*Pseudomonas syzygii*, the causal agent of Sumatra disease of cloves (*Syzygium aromaticum*), is a close relative of *B. solanacearum*, as determined by phenotypic data and DNA-DNA hybridization data (25). The blood disease bacterium (BDB), the causal agent of blood disease of bananas, affects certain members of the Musaceae in Indonesia (1, 6). The BDB strains have a distinct host range and several physiological and nutritional characteristics that are quite distinct from characteristics of *B. solanacearum* (5, 6, 28). However, partial 16S rDNA sequence data for the BDB and *P. syzygii* have confirmed that these organisms are close relatives of *B. solanacearum* (28).

Therefore, complete sequence data for the 16S rRNA genes of *P. syzygii* and the BDB could help resolve the relationships of these bacteria and *B. solanacearum*.

MATERIALS AND METHODS

Bacterial strains. The bacterial isolates used in this study are listed in Table 1. *B. solanacearum* and the BDB isolates were grown on sucrose-peptone agar (10) or tetrazolium chloride plates (16) and were incubated at 28°C for 48 to 72 h. *P. syzygii* isolates were cultured on Casamino Acids medium (25).

DNA purification. Genomic DNA was extracted and purified by using two methods, the method of Marmur (21) and the method of Buchert et al. (2).

PCR. The 16S rRNA gene was amplified in a 100-μl (total volume) reaction mixture containing PCR buffer [67 mM Tris-HCl (pH 8.8), 16.6 mM (NH₄)₂SO₄, 0.45% (vol/vol) Triton X-100, 200 μg of gelatin per ml], 1.5 mM MgCl₂, each deoxynucleoside triphosphate at a concentration of 200 μM, 0.25 μM primer 27F, 0.25 μM primer 1525R (17), 100 ng of DNA, and 1 U of Tth Plus DNA polymerase (Biotech International, Ltd., Perth, Australia). Negative controls that contained all of the ingredients described above except for the template DNA were included in each experiment. Each reaction mixture was overlaid with 80 μl of mineral oil to prevent evaporation.
The nucleotide sequences determined in this study have been deposited in the GenBank data library under the accession numbers shown in Table 1.

**TABLE 1. B. solanacearum strains and related bacteria studied**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Strain</th>
<th>Location</th>
<th>Host</th>
<th>Biovar</th>
<th>Division or subdivision</th>
<th>Year isolated</th>
<th>Other designation(s)</th>
<th>GenBank accession no.</th>
</tr>
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<tr>
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<td>R288</td>
<td>People's Republic of China</td>
<td>Mulberry</td>
<td>5 1</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>ACH092</td>
<td>Australia</td>
<td>Ginger</td>
<td>4 1</td>
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</tr>
<tr>
<td></td>
<td>ACH0171</td>
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<td>3 1</td>
<td>1967</td>
<td></td>
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<tr>
<td></td>
<td>R791</td>
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<td>Tomato</td>
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<td></td>
<td></td>
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<td>U28220</td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
<td>PDDCC1727T</td>
<td>United States</td>
<td>Tomato</td>
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<td></td>
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<td>ATCC 16967T, K60T, ACM3851T</td>
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<td>CIP 210</td>
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<td></td>
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<td>Musa ABB (Moko)</td>
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<td></td>
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<tr>
<td>Br 150</td>
<td>United Kingdom</td>
<td>Solanum dulcamara</td>
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<td>Chile</td>
<td>Potato</td>
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<tr>
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<td>Philippines</td>
<td>Banana (Bugtok)</td>
<td>1 2a</td>
<td>1991</td>
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<td>U28231</td>
</tr>
<tr>
<td></td>
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<td>Indonesia</td>
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<td>N2 2b</td>
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<td>U28232</td>
</tr>
<tr>
<td></td>
<td>R142</td>
<td>Indonesia</td>
<td>Clove</td>
<td>2 2b</td>
<td>1985</td>
<td></td>
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<tr>
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<td>P. syzygi</td>
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<td>2b</td>
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<td></td>
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* Abbreviations: R. Rothamsted Experimental Station, Harpenden, Hertfordshire, United Kingdom; ACH, A. C. Hayward, Department of Microbiology, Centre for Bacterial Diversity and Identification, The University of Queensland, St. Lucia, Australia; ACM, Australian Collection of Microorganisms, Department of Microbiology, Centre for Bacterial Diversity and Identification, The University of Queensland, St. Lucia, Australia; CIP, International Potato Center, Lima, Peru; ATCC, American Type Culture Collection, Rockville, MD; PDDCC, Culture Collection of Plant Diseases Division, New Zealand Department of Scientific and Industrial Research, Auckland, New Zealand.

RESULTS

We determined nearly complete 16S rDNA sequences for 19 isolates of B. solanacearum, three isolates of the BDB, and two isolates of P. syzygi. The dendrogram (Fig. 1) that was produced by comparing 1,422 nucleotide positions (all ambiguous nucleotides were omitted from the comparisons) contains two divisions, which is consistent with the findings of Cook et al. (3) and Li et al. (18). B. solanacearum biovar 3, 4, and 5 strains and an aberrant biovar 2 isolate (strain ACH0732) made up division 1, and B. solanacearum biovar 1, 2, and N2 strains, the BDB strains, and P. syzygi strains made up division 2. Two subdivisions were recognized within division 2, and these subdivisions were designated subdivisions 2a and 2b. Subdivision 2b comprised P. syzygi, the BDB, and two Indonesian B. solanacearum isolates, strains R780 and R142. Subdivision 2a comprised all other biovar 1, 2, and N2 isolates. As determined by bootstrap values, the branch point separating divisions 1 and 2 was well supported (100% of evolutionary distance-based trees and 92% of parsimony-based trees). However, the branch point separating the two subdivisions of division 2 was not well supported (59% of evolutionary distance-based trees and 53% of parsimony-based trees) (Fig. 1).

The sequence differences and their positions (in the Escherichia coli numbering system (35)) are shown in Table 2. Positions 458 to 460 (UUC) and 474 (A) characterized the division 2 isolates. The nucleotides at the same positions in the division 1 isolates were ACU and U, respectively, except in aberrant biovar 2 isolate ACH0732. Strain ACH0732 had a U at posi-
Akaligenes eutrophus solanaceatum were found; in each case the upper value is the percentage of distance trees in division 2 (including subdivisions 2a and 2b) the levels of sequence similarity ranged from 99.1 to 100%. Within subdivisions 2a and 2b, respectively. The levels of 16S rDNA sequence similarity between the BDB and B. solanacearum isolates ranged from 99.1 to 99.9%. The sequences of the B. solanacearum R780 and R142 isolates were most similar to the sequences of the BDB strains; all of these organisms were isolated from plants in Indonesia (Table 1). The levels of sequence similarity between the P. syzygii isolates and the B. solanacearum isolates sequenced ranged from 99.2 to 99.9%. Again, the B. solanacearum isolates which exhibited the highest levels of similarity to P. syzygii isolates were strains R780 and R142.

The sequences of strains ACH0158, ACH092, ACH0171, and PDDCC1727T determined previously by Li et al. (18) were confirmed, except that one extra G occurred at position 1456 in strains PDDCC1727T and ACH0158.

**DISCUSSION**

We determined the 16S rRNA gene sequences of 19 strains of B. solanacearum that were isolated from various hosts and geographical regions (Table 1) and represented all known biovars (10, 14). The results of our sequence analysis and comparisons expressed in the dendrogram in Fig. 1 confirm and extend the results of Li et al. (18). The two main clusters, designated divisions 1 and 2 (Fig. 1), which were identified in this study and by Li et al. (18) on the basis of 16S rDNA sequences correspond to the two divisions defined by Cook et al. (3) on the basis of restriction fragment length polymorphism analysis data. Cook et al. (3) showed that the similarity coefficient based on pairwise comparisons of all restriction fragment length polymorphism groups for groups division 1 biovars 3, 4, and 5) and division 2 (biovars 1, 2, and N2) was only 13.5%.

The divergent phylogeny observed with B. solanacearum strains corresponds to a level of DNA reassocation that is less than the level of DNA reassocation expected within a species. Strains of the same species are expected to exhibit DNA reassocation values of more than 70% (30), whereas the levels of DNA-DNA hybridization between the members of different biovars of B. solanacearum have commonly been substantially less than 70% (23, 25). However, Roberts et al. (25) and Pallaroni and Doudoroff (23) found that the techniques used to measure levels of DNA-DNA hybridization of B. solanacearum strains resulted in inherent variability (25) or produced inconsistent results (23) and may therefore only be useful as a guide to the relatedness of strains. It is evident from restriction fragment length polymorphism analysis data, 16S rRNA gene sequence data, and phenotypic differences that the two divisions of B. solanacearum may represent subspecies, as suggested by Li et al. (18), or separate species. Below we refer to the species B. solanacearum, as currently described phenotypically, as a species complex to reflect this intraspecies variation. B. solanacearum ACH0732, which clusters with biovars 3, 4, and 5 in division 1, was originally isolated from a tomato plant in Darwin, Northern Territory, Australia, in October 1978. The characteristics of this isolate are for the most part typical of B. solanacearum, as currently described phenotypically.
Strain ACH0732 produces acid from trehalose, unlike all other biovar 2 isolates found in Australia (14), and for this reason has been considered aberrant. No other biovar 2 isolates have been isolated from the Northern Territory. In Australia, biovar 2 isolates are isolated almost exclusively from potatoes or a few solanaceous weeds in southern and eastern states (11), whereas strain ACH0732 came from a lowland tropical region with no previous history of potato cultivation. In a comparison of whole-cell protein profiles, ACH0732 occupied a position intermediate between the clusters representing division 1 (biovars 3 and 4) and division 2 (biovars 1 and 2) (19). In this study we found that ACH0732 has a unique tRNA fingerprint as the BDB, but has the same fatty acid profiles (25,31), and are serologically related to biovars 3 and 4 than to biovars 1 and 2 (19). In this study we found that P. syzygii (at position 205) was confirmed in this study (Table 2). The sequence of P. syzygii which we determined differs from the sequence of Seal et al. (28) by a single base at position 204 in strain R001; this strain has a C rather than the G which is found in all other B. solanacearum and BDB isolates sequenced by us and Seal et al. (28).

We found that P. syzygii and the BDB cluster within the B. solanacearum species complex (Fig. 1). P. syzygii, the BDB, and two isolates of B. solanacearum, strains R142 and R780, form a separate cluster within division 2, designated subdivision 2b (Fig. 1), which contains only isolates from Indonesia. Isolate R780 is a biovar N2 isolate and was obtained from a potato. Island R142 was isolated from cloves, which is not a common host of B. solanacearum biovar 2 isolates. This strain is an atypical biovar 2 isolate which does not belong to race 3 and exhibits the same RNA fingerprint as the BDB, but has the host range and biochemical characteristics of B. solanacearum (27, 28).

The subgroup containing Indonesian isolates (subdivision 2b) (Fig. 1) exhibits sequence homology with division 1 isolates at positions 649, 1424, 1428, 1456, and 1472 (V7 and V5 region of Gray et al. [9]) and with subdivision 2a isolates at positions 458 to 460 and 474. Division 1 isolate ACH0732 is an exception; this isolate has the same base as division 2 isolates at position 458 and has a unique base at position 474. Furthermore, subdivision 2b isolates have two unique nucleotides, at positions 669 and 737. Although the branch point between subdivisions 2a and 2b is not stable (Fig. 1) (the bootstrap values are less than 60%), the nucleotide positions listed above unify the group. Phenotypically, the subdivision 2b organisms are divergent, and they belong to two valid species (B. solanacearum and P. syzygii) and one species (BDB) which has not been validly described, although they have very similar 16S rDNA sequences.

Despite the high levels of 16S rDNA sequence similarity...
between \textit{P. syzygii} strains and members of the \textit{B. solanacearum} species complex, recognition of \textit{P. syzygii} as a separate species is justified because it has a clearly distinct phenotype (5, 25). In contrast, the phenotype of the BDB is similar to the phenotype of \textit{B. solanacearum} (5). In a numerical analysis of more than 70 phenotypic properties, Eden-Green (5) showed that the BDB forms a distinct phenon that is intermediate between the clusters corresponding to divisions 1 and 2 of \textit{B. solanacearum}. Also, the symptoms of blood disease of banana in Indonesia are similar to the symptoms of the insect-transmitted Moko disease caused by strains of \textit{B. solanacearum} (e.g., \textit{B. solanacearum} race 2 strain SFR) (32). However, the nine nucleotide differences (Table 2, positions 649, 669, 737, 848, 1424, 1428, 1441, 1456, and 1472) between the sequences of three BDB strains and three Moko disease strains of \textit{B. solanacearum} and the phenotypic differences between these organisms provide evidence that the BDB is distinct from the \textit{B. solanacearum} species complex strains that cause Moko disease in Central and South America and The Philippines. Stover and Espinoza (32) concluded that Moko disease and blood disease of banana are closely related. Moko disease-causing \textit{B. solanacearum} strains and the BDB have independently evolved similar pathologies on jungle \textit{H. sapiens} species in Central America and probably on wild \textit{Musa} species in southern Sulawesi (Indonesia), respectively (32). In addition, our results also suggest that both the BDB and \textit{P. syzygii} evolved from the same progenitor organism as \textit{B. solanacearum}, which includes the Moko disease-causing organism. It is likely that in Indonesia host specialization resulted in the phenotypically distinct species \textit{P. syzygii} on cloves and BDB on bananas. 

Eden-Green (5) hypothesized that Bugtok disease of cooking bananas in The Philippines and Moko disease of dessert bananas are caused by the same organism. Eden-Green (5) also suggested that, as the first reports of Bugtok disease predate the reported introduction of Moko disease into The Philippines, either Moko disease was introduced into The Philippines at an earlier date or Central American strains that cause Moko disease may have originated in Southeast Asia. Our results which show that Bugtok and Moko disease isolates from The Philippines have the same 16S rDNA sequence and have a single unique nucleotide at position 1208 (Table 2) support Eden-Green's hypothesis that Moko and Bugtok disease organisms from The Philippines may be the same organism. However, we can make no conclusion concerning the similarity of the rest of the genomes of these organisms. The polymorphisms found in the 16S rDNAs of members of the \textit{B. solanacearum} species complex and the levels of 16S rDNA sequence similarity between members of the \textit{B. solanacearum} species complex and related organisms (BDB and \textit{P. syzygii}), together with the phenotypic variation among these organisms, suggest that they belong to a rapidly evolving (tachytic) group. Alternatively, it is also possible that \textit{B. solanacearum}, as defined phenotypically, represents more than one species. If this is the case, \textit{B. solanacearum} would appear to be a rapidly evolving species as the strains which we compared may actually belong to different species. Further work on the DNA-DNA relatedness of strains belonging to the \textit{B. solanacearum} species complex and related bacteria will be required to resolve this issue. The high level of variability within the \textit{B. solanacearum} species complex and related bacteria will be required to resolve this issue. The high level of variability within the \textit{B. solanacearum} species complex and related bacteria will be required to resolve this issue.

### Acknowledgments

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### References