Differentiation of *Pseudomonas solanacearum*, *Pseudomonas syzygii*, *Pseudomonas pickettii* and the Blood Disease Bacterium by partial 16S rRNA sequencing: construction of oligonucleotide primers for sensitive detection by polymerase chain reaction

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(Received 11 November 1992; revised 24 February 1993; accepted 4 March 1993)

The sequence of a 292 bp segment of the DNA encoding 16S rRNA (corresponding to positions 44–337 of the *Escherichia coli* 16S rRNA sequence) was determined for each of 40 *Pseudomonas solanacearum*, four banana Blood Disease Bacterium, three *P. syzygii* and two *P. pickettii* strains. Phylogenetic relationships derived from comparison of these sequences to each other, and to equivalent 16S rRNA gene sequences from other bacteria present in the EMBL databank, conform well with those obtained previously by DNA–DNA/rRNA hybridization experiments. The 16S rRNA sequence of the Blood Disease Bacterium was identical over the 292 bp to one of the four sequence groups of *P. solanacearum*, suggesting that these pseudomonads are more closely related to each other than to *P. syzygii* or *P. pickettii*. Sequence data comparisons allowed construction of an oligonucleotide specific for *P. solanacearum*, *P. syzygii* and the Blood Disease Bacterium. Use of the specific oligonucleotide with a non-specific oligonucleotide in the polymerase chain reaction enabled 1–10 cells of bacteria in this group to be detected after 50 rounds of amplification by visualizing a 287–288 bp product on agarose gels.

Introduction

The species *Pseudomonas solanacearum*, *P. pickettii* and *P. syzygii* fall within the same DNA–DNA homology group of *Pseudomonas* rRNA group II (De Vos & De Ley, 1983; Palleroni *et al.*, 1973; Palleroni, 1984; Roberts *et al.*, 1990), which contains many pathogenic pseudomonads. *P. solanacearum* causes bacterial wilt of a large range of crops in the tropics, subtropics and warm temperate regions of the world. *P. pickettii* and *P. syzygii* are closely related species (Ralston *et al.*, 1973; Roberts

et al., 1990) which cause opportunistic infections in humans and Sumatra disease of cloves (*Syzygium aromaticum*), respectively. Blood Disease Bacterium (BDB) strains affect certain members of the *Musaceae* in Indonesia (Gäumann, 1923; Eden-Green & Sastraatmadja, 1990), giving rise to symptoms similar to bacterial wilt of banana (termed Moko disease), but with pronounced exudation of a reddish-coloured bacterial ooze. Whether the BDB should be classified as a separate species (e.g. '*P. celebense*' as originally proposed by Gäumann, 1923) or as a new subgroup of *P. solanacearum* remains unresolved. BDB strains, like *P. syzygii* (and to a more limited extent *P. pickettii*), are related to *P. solanacearum* serologically as well as by DNA–DNA homology (Roberts *et al.*, 1990; Hayward, 1991; Cook *et al.*, 1991), and show similar fingerprints after polymerase chain reaction (PCR) amplification with tRNA consensus primers (Seal *et al.*, 1992c). They are, however, easily differentiated by host range, cultural and physiological properties (Eden-Green & Sastraatmadja, 1990).

P. solanacearum strains represent a heterogeneous group that has been subdivided into five races based on host range, and five biovars based on catabolic properties

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Abbreviations: PCR, polymerase chain reaction; BDB, Blood Disease Bacterium; RFLP, restriction fragment length polymorphism; rDNA, DNA encoding ribosomal RNA.

The nucleotide sequence data reported in this paper have been submitted to EMBL and have been assigned the accession numbers X70344–X70350.

Table 1. *Bacterial strains*

Strain	Race*	Biovar†	Origin‡	Contributor§	Group
<i>Pseudomonas solanacearum</i>					
UW25	1	1	Tomato, USA, Kelman K60	W	3
UW26	1	1	Tomato, USA, Kelman K74	W	3
UW256	1	1	Potato, Costa Rica, Gonzalez G-7	W	2
UW275	1	1	<i>Melampodium perfoliatum</i> , Costa Rica, Sequeira	W	2
UW278	1	1	Tobacco, Mexico, Fucikovsky	W	2
B9043	1	1	Clove, Indonesia, Eden-Green	R	4
R142		2	Clove, Indonesia, Eden-Green S710	R	4
R361		N2	Potato, Peru, Turkensteen CIP61	R	1
R568		N2	Potato, Brazil, Neto 172, CIP226	R	1
R578		N2	Eggplant, Peru, Aley SR130	R	1
R583		N2	Potato, Peru, Martin CIP172	R	1
S825	1	3	Clove, Indonesia, Eden-Green	R	1
S925	1	3	Clove, Indonesia, Eden-Green	R	1
T451	1	3	<i>Zingiber cassumunar</i> , Indonesia, Supriadi	B	1
T456	1	3	Castor bean, Indonesia, Supriadi	B	1
T496	1	3	<i>Croton</i> , Indonesia, Subandiah 1605WS	B	1
UW8	1	3	<i>Eupatorium odoratum</i> , Costa Rica, Sequeira K201	W	1
UW147	1	3	Tobacco, Australia, Hayward S240	W	1
UW380	1	3	Olive, China, He OPS2	W	1
GMI1000	1	4	Tomato, Guyana, Boucher <i>et al.</i> (1985)	A	1
PD1682	1	4	Ginger, Thailand, Sardud	J	1
R27	1	4	Potato, Indonesia, Hayward B050	R	1
UW74	1	4	Potato, Sri Lanka, CMI #B2861	W	1
UW151	1	4	Ginger, Australia, Hayward S244	W	1
UW378	1	4	Olive, China, He OPS1	W	1
UW361	1	5	Mulberry, China, He MPS4	W	1
UW373	1	5	Mulberry, China, He MPS2	W	1
UW160	2	1	Plantain, Peru, Sequeira S253	W	2
UW167	2	1	Banana, Costa Rica, Sequeira K160	W	1
GMI8133	2	1	Plantain, Colombia, Thurston S210	A	3
JEBUG2	2A	1	Banana, Philippines, Elphinstone	R	1
JEMOK7	2	1	Banana, Philippines, Elphinstone	R	1
R207	2	1	Banana, Belize, Black ARI-S	R	2
R273	2	1	Banana, Trinidad, Rajnauth SFR1	R	2
R484	2A	1	Banana, Philippines, Eden-Green P14	R	1
R570	2	1	Plantain, Costa Rica, CIP19, UW155	R	1
K46	3	2	Potato, Kenya, Forde	R	1
P2	3	2	Potato, Philippines, Seal	D	1
UW19	3	2	Potato, Colombia, Thurston S205	W	1
UW23	3	2	Potato, Israel, Volcani K56	W	1
Blood disease bacterium					
R229			Banana, Indonesia, Eden-Green T389	R	4
R230			Banana, Indonesia, Eden-Green T334	R	4
R231			Banana, Indonesia, Eden-Green T391	R	4
T340			Banana, Indonesia, Eden-Green	R	4
<i>Pseudomonas pickettii</i>					
E1625			Soil/potato, Ethiopia, Wondimagegne	E	
NC11149				N	
<i>Pseudomonas syzygii</i>					
R001			Clove, Indonesia, Eden-Green	R	
R004			<i>Hindola fulva</i> , Indonesia, Eden-Green	R	
R170			Clove, Indonesia, Eden-Green	R	

* 2A, bugtok strains.

† N2, new biovar 2 (lowland strains).

‡ Host, country, isolator and alternative strain designation are given.

§ Strains were contributed by: A, M. Arlat & P. Barberis, CNRS-INRA, Auzeville, Castanet-Tolosan Cedex, France; B, Supriadi, Balai Penelitian Tanaman Rempah dan Obat, Jl. Cimanggu 3, Bogor, Indonesia; D, M. Daniels & S. Seal, The Sainsbury Laboratory, Norwich, UK; E,

(see Hayward, 1991). Inconsistencies in DNA–DNA homologies of strains of *P. solanacearum* with each other have been noted by Palleroni & Doudoroff (1971) and by Roberts *et al.* (1990). It was suggested by Roberts *et al.* (1990) that the relatedness between strains of *P. solanacearum* is less than that which would be expected within a species. Seal *et al.* (1992c) found that *P. solanacearum* strains gave rise to three ‘fingerprint’ groups after PCR amplification with tRNA consensus primers. Welsh & McClelland (1991) found that these primers generated PCR fingerprints which showed little variation within a species and between closely related species. The division of *P. solanacearum* strains into these three PCR fingerprint groups conforms well to the major divisions made in the 33 RFLP groups by Cook *et al.* (1991) (Seal *et al.*, 1992c). Further studies of the taxonomic relationships within a large number of strains of *P. solanacearum* are required to clarify relationships of subgroups of *P. solanacearum* to each other, to the BDB, and to *P. syzygii*.

Traditionally, classification of bacteria has been based on similarities in phenotypic characteristics. However, classification based on these criteria does not necessarily correlate well with evolutionary relationships defined by macromolecular comparisons (Woese, 1987). Of the macromolecules used for phylogenetic analyses, the 16S rRNA molecule has become particularly valuable in recent years for ascertaining the interrelationships of bacteria (Woese, 1987). This is not only because of its high information content, conservative nature and universal distribution, but also due to the rapidly expanding 16S rRNA sequence data accessible through gene databanks that can be used for phylogenetic analysis. With the development of the PCR and techniques for the direct sequencing of amplified DNA (Innes *et al.*, 1988; Winship, 1989), rRNA-encoding DNA (rDNA) sequences can now be obtained relatively quickly. The aim of this study was to ascertain the phylogenetic relationships of subgroups within *P. solanacearum* to each other and to closely related BDB strains, *P. syzygii* and *P. pickettii*. To this end, primers designed by Young *et al.* (1991), which amplify a ~300 bp fragment of most bacterial 16S rDNAs, were used. The amplified region represents a relatively variable part of the 16S rRNA and thus has a high density of information, especially for comparisons of close relatives (Young *et al.*, 1991). Phylogenetic relationships between

species of the alpha proteobacteria deduced from this part of the molecule are similar to those derived from sequences of the entire 16S rRNA molecule (Young *et al.*, 1991).

Sequences of 16S rDNA genes are often species-specific and are present as multiple copies in microbial genomes. Hence they make excellent targets for identification of bacteria at the species level. Hybridization with short oligonucleotide labelled probes to species-specific sequences within the 16S rRNA molecule is used often as an identification procedure (Amann *et al.*, 1990; DeLong *et al.*, 1989; Giovannoni *et al.*, 1988; Zarda *et al.*, 1991). Recently, the corresponding specific rDNA sequences have been used as the targets for PCR amplification with complementary oligonucleotide primers. Amplification in the order of a million-fold can be achieved using PCR technology, which has resulted in specific detection of a single bacterial cell becoming feasible. Diagnostic tests based on specific PCR amplification of plant pathogens from environmental samples have shown that rapid detection of very low numbers of the pathogen can be achieved without the need for culturing (Ahrens & Seemüller, 1992; Bereswill *et al.*, 1992; Schesser *et al.*, 1991; Seal *et al.*, 1992b). Rapid, highly sensitive diagnostic tests for *P. solanacearum* are required for quarantine and planting material certification purposes, as this pathogen can infect potato, tomato, ginger and banana without inducing symptoms.

In this paper we report the construction of a primer specific to the 16S rDNA of *P. solanacearum*, the BDB and *P. syzygii*, which enables more sensitive detection by PCR amplification of *P. solanacearum* in culture and potato tubers than previously achieved with ELISA, nucleic acid probes and PCR primers (Seal *et al.*, 1992a, b).

Methods

Bacterial strains. The characteristics of the strains tested in this study are shown in Table 1.

Preparation of PCR-amplifiable DNA. All strains were grown overnight in NYGB (Daniels *et al.*, 1984) at 28 °C with shaking, and bacterial DNA was isolated by the method of Boucher *et al.* (1987), which involves lysis with Sarkosyl, proteinase K treatment, phenol/chloroform extraction and ethanol precipitation. Alternatively, whole *P. solanacearum* cells could be used to provide a template for PCR amplification, but the cultures had to be grown on MM minimal

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|| *P. solanacearum* sequencing group.

medium (Boucher *et al.*, 1985) to ensure adequate lysis of the cells. Lysis was performed by boiling 100 µl of an overnight MM culture (or a loopful of cells from a colony resuspended in 100 µl sterile distilled water) for 5 min. After cooling to room temperature 2 µl of the boiled culture was used per reaction without further treatment.

To determine the sensitivity of PCR detection, 100 µl samples of 10-fold dilutions of exponentially growing MM cultures were plated out on NYGA (Daniels *et al.*, 1984), incubated at 28 °C for 2–3 d and colonies counted. Samples (100 µl) of the 10-fold dilutions of MM cultures were also boiled as described above, and aliquots used for PCR amplification. This enabled a calculation of the approximate number of lysed boiled cells added to the PCR reactions.

PCR amplification. The primers used were Y1 and Y2, designed by Young *et al.* (1991) to amplify a partial segment (corresponding to positions 40–337 of *Escherichia coli* 16S rRNA) of most DNAs encoding 16S rRNA. PCR amplifications were performed using DNA thermal cyclers (Perkin Elmer Cetus). Reaction volumes were 100 µl and contained 1 × PCR buffer [10 mM-Tris/HCl pH 8.3 at 25 °C, 50 mM-KCl, 1.5 mM-MgCl₂, 0.001% (w/v) gelatin (Sigma G-2500), 0.05% (v/v) Nonidet P40 (Sigma N-3516), 0.05% (v/v) Tween 20 (Sigma P-1379)], 0.2 mM of each dNTP, 1 U *Taq* polymerase, 1 µM of primers Y1 (5'TGGCTCAGAACGACGCGCGGC3') and Y2 (5'CCCACTGCTGCCTCCCGTAGGAGT3'), and template DNA (either 10–20 ng of purified DNA or 2 µl of boiled MM-culture). Each reaction was overlaid with two drops of light mineral oil (Sigma M-3516), and heated to 96 °C for 2 min. Reactions were then cycled 35 times through phases of denaturation (94 °C for 20 s), annealing (61 °C for 20 s) and extension (72 °C for 30 s), followed by a final period of 10 min at 72 °C. Samples (5 µl) of reaction mixtures were examined by electrophoresis using 2% (w/v) agarose gels (Sigma A-6013) and bands revealed by staining with ethidium bromide.

For specific PCR amplification of samples containing DNA of the *P. solanacearum*, *P. syzygii* and BDB group, the non-specific primer Y2 was used together with a specific primer OLI1 (5'GGGGGTAG-CTTGCTACCTGCC3'). Reactions were as described above using 1 µM of each primer Y2 and OLI1, and 50 µl reaction volumes. Reaction mixtures were cycled in a similar fashion to Y1–Y2 samples, but an annealing temperature of 68 °C was used. For samples containing approximately less than 100 *P. solanacearum*, *P. syzygii* or BDB cells, the number of amplification rounds was increased from 35 to 50 cycles. Reactions containing *P. solanacearum*, *P. syzygii* or BDB DNA gave a single product of 288 bp (287 bp for *P. syzygii*), visible when 15 µl of the 50 µl reaction was examined by electrophoresis.

DNA sequencing. Products generated by PCR amplification with primers Y1 and Y2 were purified by using Qiagen 'tip-5' columns (Diagen, Düsseldorf, Germany) according to the manufacturer's protocol. Purified products were sequenced in both directions using the method and c⁷dITP reagents of a T7 sequencing kit (Pharmacia LKB), with the exception that the template was denatured together with the primer by boiling followed by rapid cooling in dry ice. Sequences were aligned with each other and with 16S rRNA databank sequences of other alpha and beta subgroup bacteria, using the Wisconsin programs (Devereux *et al.*, 1984). All positions where there were base changes or other mismatches were checked by repeating the sequencing of a PCR reaction product from an independent DNA preparation of the relevant strain.

Results and Discussion

Sequence data for all strains are shown in Fig. 1. Alignments can be made reliably due to high conservation of several positions and the secondary structure of the 16S rRNA molecule (Woese, 1987). It is clear from

the sequence and its predicted secondary structure (Fig. 2) that the species *P. solanacearum*, *P. syzygii*, *P. pickettii* and the BDB belong to the beta subdivision of the purple bacteria; e.g. a U at position 7 (corresponding to *E. coli* position 50) and the stem-loop between positions 152 and 175 are characteristic of the beta subdivision (Woese, 1987).

PCR amplification with primers Y1 and Y2 yielded a product of 292 bp for all 40 *P. solanacearum* and four BDB strains, a 291 bp product for the three *P. syzygii* strains and a 294 bp product for the two *P. pickettii* strains studied. Generally, there is very little variation in the 16S rRNA sequence of strains within a species (Woese, 1987). However, within the 292 bp amplified from *P. solanacearum* strains, there were two variable sites I and II (positions 122 and 224 respectively; Fig. 2), each of which showed one of two possible bases. This resulted in the 40 *P. solanacearum* strains studied falling into four sequence groups: group 1 has an A at position I and a C at position II, group 2 has an A and T, group 3 a G and T, and group 4 a G and C respectively. The group of each strain sequenced is shown in Table 1. The sequence data thus support other nucleic-acid-based studies of *P. solanacearum* (Cook *et al.*, 1989, 1991; Palleroni & Doudoroff, 1971; Roberts *et al.*, 1990; Seal *et al.*, 1992c) that show it to be a heterogeneous species.

The groups formed within *P. solanacearum* by 16S rRNA sequencing are in general agreement with those based on RFLP analysis (Cook *et al.*, 1989, 1991) and by PCR amplification with tRNA consensus primers (Seal *et al.*, 1992c), although the subdivision of biovar 1 and 2 strains into groups is not identical for these three classification methods. That is, the RFLP groups within RFLP division II (biovar 1 and 2 strains) belong to either tRNA fingerprint type I or II, and rRNA sequence groups 1, 2, 3 or 4. Biovar 3, 4 and 5 strains belong to RFLP division I, tRNA fingerprint type III, and form part of 16S rRNA sequence group 1. Although these three recent methods of classification of *P. solanacearum* strains do not support exactly the same grouping of strains, they all divide the species very differently from the traditional race classification system (Buddenhagen *et al.*, 1962), in which the quite different members of biovars 1, 3 and 4 are placed within a single race. The three methods of classification based on nucleic acid properties are consistent with a common Australasian evolutionary origin of biovars 3, 4 and 5, but uncertainty remains on evolutionary relationships within biovar 1 and 2 strains of American origin.

The sequence of the BDB rRNA is identical over the 292 bp to that of *P. solanacearum* group 4, confirming that this is a very close relative of *P. solanacearum* as previously suggested (Eden-Green & Sastraatmadja, 1990; Cook *et al.*, 1991). Group 4 contains only two *P.*

	1					60
<i>P.solan.1</i>	ATGCCTTACA	CATGCAAGTC	GAACGGCAG	.CGGGGGTAG	CTTG.CTACC	TGCCGGCGAG
<i>P.solan.2</i>	-----	-----	-----	.-----	-----	-----
<i>P.solan.3</i>	-----	-----	-----	.-----	-----	-----
<i>P.solan.4</i>	-----	-----	-----	.-----	-----	-----
<i>P.syzygii</i>	-----	-----	-----	.-----	-----	-----
<i>P.pick.1</i>	-----	-----	-----	C AT-ATCTAGC	TTGC.TAGAT	--AT-----
<i>P.pick.2</i>	-----	-----	-----	C AT-ATCTAGC	TTGC.TAGAT	--AT-----
<i>A.eutrop.</i>	-----	-----	-----	C A---CT... .	.T.-GG-	--GT-----
<i>P.cepacia</i>	-----	-----	-----	C ATC---... .	TGCTTNC-	-NGT-----
	61					120
<i>P.solan.1</i>	TGGCGAACGG	GTGAGTAATA	CATCGGAACG	TGCCCTGTAG	TGGGGGATAA	CTAGTCGAAA
<i>P.solan.2</i>	-----	-----	-----	-----	-----	-----
<i>P.solan.3</i>	-----	-----	-----	-----	-----	-----
<i>P.solan.4</i>	-----	-----	-----	-----	-----	-----
<i>P.syzygii</i>	-----	-----	-----	-----	-----	-----
<i>P.pick.1</i>	-----	-----	-----	-----	-----	-----
<i>P.pick.2</i>	-----	-----	-----	-----	-----	-----
<i>A.eutrop.</i>	-----	-----	-----	-----	-----	-----
<i>P.cepacia</i>	-----	-----	-----	A --T-----	-----G	-C.-G-----
	121					180
<i>P.solan.1</i>	GACTAGCTAA	TACCGCATAC	GACCTGAGGG	TGAAAGTGGG	GGACCGCAAG	GCCTCATGCT
<i>P.solan.2</i>	-----	-----	-----	-----	-----	-----
<i>P.solan.3</i>	---G-----	-----	-----	-----	-----	-----
<i>P.solan.4</i>	---G-----	-----	-----	-----	-----	-----
<i>P.syzygii</i>	---G-----	-----	-----	-----	-----	-----
<i>P.pick.1</i>	--T-----	-----	-----	-----	-----	-----
<i>P.pick.2</i>	--T-----	-----	-----	-----	-----	-----
<i>A.eutrop.</i>	--T-----	-----	-----	-----C-----	-----N-----	-----GC-----
<i>P.cepacia</i>	-C-NNAT---	-----	--T--AC--A	-----C---	N---TTCG-	-----GC-----
	181					240
<i>P.solan.1</i>	ATAGGAGCGG	CCGATGTCTG	ATTAGCTAGT	TGGTGGGGTA	AAGGCCCAAC	AAGGCGACGA
<i>P.solan.2</i>	-----	-----	-----	-----	-----T-----	-----
<i>P.solan.3</i>	-----	-----	-----	-----	-----T-----	-----
<i>P.solan.4</i>	-----	-----	-----	-----	-----	-----
<i>P.syzygii</i>	-----	-----	-----	-----	-----	-----
<i>P.pick.1</i>	-----	-----	-----	-----	-----	-----
<i>P.pick.2</i>	-----	-----	-----	-----A-----	-----T-----	-----
<i>A.eutrop.</i>	-C-----	-----	-----	-----	-----T-----	-----
<i>P.cepacia</i>	-----GTT-----	-----G-----	-----	-----	-----T-----	-----
	241					295
<i>P.solan.1</i>	TCAGTAGCTG	GTCTGAGAGG	ACGATCAGCC	ACACTGGGAC	TGAGACACGG	CCCAG
<i>P.solan.2</i>	-----	-----	-----	-----	-----	-----
<i>P.solan.3</i>	-----	-----	-----	-----	-----	-----
<i>P.solan.4</i>	-----	-----	-----	-----	-----	-----
<i>P.syzygii</i>	-----	-----	-----	-----	-----G-----	-----
<i>P.pick.1</i>	-----	-----	-----	-----	-----	-----
<i>P.pick.2</i>	-----	-----	-----	-----	-----	-----
<i>A.eutrop.</i>	-----	-----	-----	-----	-----	-----
<i>P.cepacia</i>	-----N-----	-----	-----C-----	-----	-----	-----N-----

Fig. 1. Aligned sequences of part of the 16S rRNA gene of strains of *P. solanacearum* (*P. solan.* 1 to *P. solan.* 4) (see Table 1 for strains in sequence groups 1 to 4), Blood Disease Bacterium (*P. solan.* 4), *P. syzygii*, *P. pickettii* (*P. pick.* 1 and *P. pick.* 2; strains E1625 and NC11149, respectively), *Alcaligenes eutrophus* (*A. eutrop.*), and *Pseudomonas cepacia*. The sequences shown represent the region amplified by primers Y1 and Y2, but exclude the primer sequences. Dashes indicate that the base is identical to that found in *P. solanacearum* group 1 (*P. solan.* 1). Dots indicate alignment gaps. The sequences *A. eutrop.* and *P. cepacia* are shown for comparison, and represent part of sequences AERGSSA and PCRRDA respectively, which are present in the EMBL DataBank.

solanacearum strains (B9043 and R142) of the 40 sequenced. Both these strains were isolated from clove in Indonesia and are atypical in other characteristics: B9043 is a race 1 biovar 1 strain – such strains are not commonly isolated in Asia; and R142 is a biovar 2 strain but not of race 3 (to date, biovar 2 strains in Asia have

been isolated only from potato and all were race 3 strains). Strains B9043 and R142 also show the same fingerprint type as the BDB when amplified with tRNA consensus primers (Seal *et al.*, 1992c), but have nutritional properties and a host range typical of *P. solanacearum*. The similarity in 16S rRNA sequence and

Fig. 2. Predicted secondary structure of the part of the 16S rRNA molecule encoded by the sequence determined for *P. solanacearum*. The secondary structure was developed from that published for *E. coli* (Woese, 1987). Numbers prefixed by 'Ec' are corresponding positions in the complete *E. coli* sequence. Arabic numbers indicate the approximate position in the sequence alignment of Fig. 1. Roman numerals indicate the positions that are variable in the *P. solanacearum*, *P. syzygii* and BDB strains studied. The sequence immediately adjacent to primer Y1 interacts with parts of the molecule not shown.

tRNA PCR fingerprints suggests that it may not be appropriate to consider the BDB as a separate species. However no 'Moko' strain falls into the same 16S rRNA sequence group as the BDB strains, and hence it may be worthwhile to preserve the distinction between these very similar pseudomonads that cause vascular infections of *Musaceae*. Sequencing more of the 16S rRNA molecule may clarify the taxonomic status of the BDB, although taxonomy should not be based on 16S rRNA sequence data alone. DNA from the BDB hybridized to some *P. solanacearum* DNA probes (Cook *et al.*, 1991; Cook & Sequeira, 1991), but not to many other probes, some of which appear to hybridize to sequences which are highly conserved in *P. solanacearum* strains (Seal *et al.*, 1992b; S. E. Seal, unpublished results). Moreover, BDB strains have a different host range, and possess several physiological and nutritional characteristics quite distinct from *P. solanacearum* (Eden-Green & Sastraatmadja, 1990; S. Eden-Green, unpublished results). A more detailed

study, based on as wide a range of characteristics as possible, is required to resolve the relationship between the BDB and *P. solanacearum*.

P. syzygii shows a similar 16S rRNA sequence to *P. solanacearum* group 4, but is one base shorter in length (the base at position III in Fig. 2 is missing), and has a G rather than a C at position IV (Fig. 2). However, *P. pickettii* differs considerably from *P. solanacearum* and *P. syzygii*, notably between positions 30 and 54. The two strains of *P. pickettii* used also differ from one another at positions 216 and 226. In separate studies, both *P. syzygii* and *P. pickettii* were shown to be close relatives of *P. solanacearum* (Roberts *et al.*, 1990; Ralston *et al.*, 1973). Partial 16S rRNA sequence data suggest that *P. syzygii* is phylogenetically closer to *P. solanacearum* than *P. pickettii* is, although an unambiguous phylogenetic tree cannot be determined from comparison of less than 300 bases of the 16S rRNA molecule. Both species showed greater sequence homology to *P. solanacearum* than *Alcaligenes eutrophus* and *Pseudomonas cepacia* (the sequences of which are included in Fig. 1 for comparison), which were previously shown to be related by DNA-rRNA hybridization studies (De Vos & De Ley, 1983).

Two of the 40 *P. solanacearum* strains studied, JEBUG2 and R484, are strains that cause 'bugtok' disease of cooking bananas in the Philippines. Bugtok disease was first reported by Roperos (1965) and is similar to Moko disease, except that symptoms are generally confined to the floral raceme and foliar symptoms are absent. Partial 16S rRNA sequence data support the inclusion of the bugtok bacterium within the species *P. solanacearum*, as suggested by cultural characteristics and RFLP patterns (S. E. Seal, unpublished results).

An oligonucleotide specific for the group *P. solanacearum*, *P. syzygii* and the BDB was constructed by comparing the partial 16S rRNA gene sequences with equivalent sequences for other organisms present in the EMBL databank. Use of this specific primer, OLI1, together with the non-specific primer Y2 in PCR with an annealing temperature of 68 °C allowed specific amplification of a single 287–288 bp product. No visible PCR product was generated from strains of the closely related species *P. pickettii*, nor from strains of *A. eutrophus*, *P. cepacia* or 12 other bacterial species (data not shown). All 59 strains of the group *P. solanacearum*–*P. syzygii*–BDB tested, including 19 strains for which rRNA sequence data were not obtained, produced the specific PCR product.

To determine the limit of detection of PCR amplification with primers Y2 and OLI1, 5 µl samples of boiled 10-fold dilutions of exponentially growing cultures of three strains were used as a template for the PCR.

Fig. 3. Electrophoretic analysis of PCR-amplified product from boiled cells of two *P. solanacearum* strains, UW19 and UW25, using the primer pair OLI1 and Y2 and 50 cycles of amplification. The size of the PCR product is ~ 300 base pairs. Tracks are labelled with the approximate number of cells used as template for the PCR.

After electrophoresis of 15 μ l aliquots of the PCR reactions (after 50 amplification rounds) the specific product could be seen easily on agarose gels from samples containing DNA from as few as six, five and two cells for strains UW19, UW25 and UW167 respectively (Fig. 3 and data not shown). The specific product could often be seen with a further 10-fold dilution of the bacterial samples, but this was not always reproducible. These numbers will be a slight overestimate of the detection sensitivity, as they have been derived from calculations of viable counts, and exponential cultures will contain DNA of both viable and non-viable cells. Nonetheless, the sensitivity achieved by this PCR detection test for *P. solanacearum* is greater than that achieved with the *P. solanacearum*-specific primer combination PS96-H/PS96-I (Seal *et al.*, 1992*b*), which has a detection limit in the range of 10^1 to 10^2 cells, depending on the strain. Crude preparations from infected potato, tomato, ginger, eggplant and pepper plants have been tested by PCR with both primer pairs. The PCR tests successfully detected *P. solanacearum* in all samples known to be infected (Seal *et al.*, 1992*a*). Detection of *P. solanacearum* in field samples by PCR using primer combination OLI1-Y2 was more sensitive than using primers PS96-H and PS96-I, some samples only generating a visible PCR product with the former primers (unpublished results). However, PCR amplification with primers OLI1 and Y2 is not specific for *P. solanacearum*, products also being produced from samples containing either *P. syzygii* or BDB DNA. In practice, this should not diminish the usefulness of this PCR test as a

diagnostic procedure for *P. solanacearum*, as *P. syzygii* and the BDB are only recorded at present in clove and banana in Indonesia. Alternatively, samples could be tested separately with both primer pairs to determine if the samples contained *P. solanacearum*, *P. syzygii* or BDB DNA. The host of origin of the sample will allow differentiation of *P. syzygii* and BDB, neither of which produces the *P. solanacearum*-specific PCR product with primers PS96-H and PS96-I.

The OLI1-Y2 PCR test will be a useful tool for investigating various questions regarding the epidemiology of the diseases caused by *P. solanacearum*, *P. syzygii* and the BDB which have remained unanswered due to the lack of a rapid sensitive test. The test should also permit more critical studies on the ecology of *P. syzygii*, which in the past have been hindered because the organism is slow growing and difficult to culture.

Latent bacterial wilt infection of symptomless potato tubers in Burundi was demonstrated, using the primer combination OLI1-Y2 and 50 PCR amplification rounds (Seal *et al.*, 1992*a*). This PCR test may have application in quarantine laboratories in countries where *P. solanacearum* is not present. The test can be completed in less than 5 h, and therefore has a great advantage over the traditional methods which involve cultivation of the bacteria. Speed is essential for quarantine laboratory assessment to limit the time that shipments wait at ports of entry. Careful inspection of potato tubers for *P. solanacearum* infection is important not only for tropical and subtropical countries, but also for temperate countries, where bacterial wilt can cause significant

losses to potato crops, as illustrated in Sweden (Olsson, 1976) and recently in Great Britain (Walker, 1992).

We would like to thank Maggie Knox and Helen Downer for providing primers and protocols for the sequencing. This work formed part of project X0082, commissioned by the Natural Resources Institute, and was carried out under MAFF licence nos PHF 1185A/99(81), PHF 1185B/10(111) and PHF 1185A/62(11), under the Plant Health (Great Britain) Order, 1987. The Sainsbury Laboratory is supported by the Gatsby Charitable Foundation.

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