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A Highly Selective PCR Protocol for Detecting 16S rRNA Genes of the Genus *Pseudomonas* (Sensu Stricto) in Environmental Samples

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***Pseudomonas* species are plant, animal, and human pathogens; exhibit plant pathogen-suppressing properties useful in biological control; or express metabolic versatilities valued in biotechnology and bioremediation. Specific detection of *Pseudomonas* species in the environment may help us gain a more complete understanding of the ecological significance of these microorganisms. The objective of this study was to develop a PCR protocol for selective detection of *Pseudomonas* (sensu stricto) in environmental samples. Extensive database searches identified a highly selective PCR primer pair for amplification of *Pseudomonas* 16S rRNA genes. A protocol that included PCR amplification and restriction analysis, a general cloning and sequencing strategy, and phylogenetic analyses was developed. The PCR protocol was validated by testing 50 target and 14 nontarget pure cultures, which confirmed the selectivity to 100%. Further validation used amplification of target sequences from purified bulk soil DNA followed by cloning of PCR products. Restriction analysis with *Hae*III revealed eight different fragmentation patterns among 36 clones. Sequencing and phylogenetic analysis of 8 representative clones indicated that 91.7% of the products were derived from target organisms of the PCR protocol. Three patterns, representing only 8.3% of the 36 clones, were derived from non-*Pseudomonas* or chimeric PCR artifacts. Three patterns, representing 61.1% of the clones, clustered with sequences of confirmed *Pseudomonas* species, whereas two patterns, representing 30.6% of the clones, formed a novel phylogenetic cluster closely associated with *Pseudomonas* species. The results indicated that the *Pseudomonas*-selective PCR primers were highly specific and may represent a powerful tool for *Pseudomonas* population structure analyses and taxonomic confirmations.**

The genus *Pseudomonas* includes species with functions of ecological, economic, and health-related importance. Some species are pathogenic for plants (8, 42), while others are opportunistic pathogens of animals or humans (10, 33, 45). Some species exhibit plant growth-promoting and pathogen-suppressing functions and may be exploited for use in biological control (18, 31). A prominent property of some species or strains is their metabolic versatility, making them attractive candidates for use in bioremediation (35, 39). Many studies have described the potential of *Pseudomonas* species to degrade a variety of compounds (12, 14, 15, 17, 41). A specific, culturing-independent detection protocol for *Pseudomonas* would represent a valuable tool in ecological and diagnostic studies of this genus. Recent advances in molecular ecological techniques and taxonomy open ways to design highly specific PCR protocols, especially for detection of 16S (small-subunit [SSU]) rRNA genes (4, 11, 29, 49). Genus-specific 16S rRNA gene PCR primer design depends on both a well-defined molecular taxonomy and a representative collection of target sequences. Each of these issues has been extensively addressed for the genus *Pseudomonas* in the last few years (27, 28, 35).

The genus *Pseudomonas*, which was described by Migula in 1894 as a genus of gram-negative, rod-shaped microorganisms (32), has been subject to repeated taxonomic revisions (35). DNA-RNA hybridization techniques have revealed five RNA classes among these pseudomonads (24, 32, 36). This extensive degree of heterogeneity among the old genus of *Pseudomonas* is reflected by the presence of distantly related species which have since been placed in existing or newly defined genera. Former *Pseudomonas* species of RNA groups II to IV have been reclassified to genera including *Brevundimonas*, *Sphingomonas*, *Burkholderia*, *Ralstonia*, *Aminobacter*, *Comamonas*, *Acidovorax*, *Hydrogenophaga*, *Telluria*, and *Stenotrophomonas* (16, 19, 35). These analyses and taxonomic rearrangements identified the RNA group I species, including the type species *P. aeruginosa* and other species such as *P. fluorescens*, *P. putida*, and *P. syringae*, as members of a phylogenetically homogeneous group referred to as *Pseudomonas* (sensu stricto) (28, 34). This classification is in agreement with phylogenetic information obtained from 16S rRNA sequence data as presented in the Ribosomal Database Project (RDP [27]). Molecular taxonomy based on 16S rRNA sequences places the genus *Pseudomonas*, which has been referred to as the type I or fluorescent group of pseudomonads, in the group called *Pseudomonas* and relatives, along with the subgroups of *Acinetobacter* and *Teredinibacter*. Directly adjacent phylogenetic branches are formed by the *Oceanospirillum* group and the *Colwellia* assem-

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blage. This molecular definition of the genus *Pseudomonas* may be used to develop a 16S rRNA-based detection approach specific for this genus.

The multitude of important characteristics of *Pseudomonas* species has inspired numerous taxonomic studies, which have led to the development of a better understanding of this genus. The approaches used include selective culturing (38), PCR amplification of rRNA genes (21, 45) in conjunction with restriction fragment length polymorphism (RFLP) analysis (5, 25) or DNA sequencing (28), DNA and RNA hybridization (3, 6, 36, 40), protein (7, 46) and fatty acid (42, 47) analyses, carbon source utilization properties (13, 17, 22), and specific antibodies (23). These techniques have been applied to characterize *Pseudomonas* species of both defined cultures and uncharacterized isolates obtained from a variety of sources. However, these approaches have not offered a rapid approach to specifically monitor and characterize population structures of the genus *Pseudomonas* (sensu stricto) in the environment.

The objective of this study was to develop a 16S rRNA gene-based PCR protocol for the selective detection of *Pseudomonas* (sensu stricto) in environmental samples. Sequence information of 16S rRNA sequences available from RDP and GenBank was compiled and searched for *Pseudomonas*-selective PCR (Ps-PC) primer sequences. Theoretical evaluation of a highly selective PCR primer pair is presented here, together with experimental validation performed on pure cultures and bulk DNA extracted from an agricultural soil sample.

MATERIALS AND METHODS

Bacterial cultures and soil DNA. Experimental specificity testing of PCR primers was performed with pure bacterial cultures obtained from the American Type Culture Collection (ATCC; Rockville, Md.), culture DNA samples (kindly provided by B. Bochner, Biolog Inc., Hayward, Calif.), cultures obtained from the Environmental Protection Agency (EPA; Corvallis, Oreg.) culture collection, and environmental isolates from the Corvallis, Oreg., area. Information on the cultures used in this study is summarized in Table 1. Selected environmental isolates from a variety of studies were identified with the Biolog microbial identification system according to the manufacturer's recommendations.

Bulk DNA extracted from an agricultural field soil, purified (37, 50), and spectrophotometrically (Spectrometer Lambda 2S; Perkin-Elmer, Norwalk, Conn.) quantified as 32.9 to 34.0 μg/g (dry weight equivalent) was kindly provided by L. A. Porteous (U.S. EPA). The field soil (Hyslop Agricultural Field Laboratory, Oregon State University, Corvallis) was classified as Woodburn silt loam and was composed of 72.2% silt, 21.5% clay, 6.3% sand, and 6.3% organic matter.

Primer design. RDP release 5 (May 1995) was used with a total of 2,849 prokaryotic entries, 13 of which were classified as members of the genus *Pseudomonas* (27). Specific alignments of 16S rRNA sequences were retrieved by the SUB_ALIGNMENT routine from the RDP (27), and potential PCR primer target regions were manually determined. Their specificity was theoretically evaluated by the CHECK_PROBE routine of the RDP by using the total prokaryotic SSU rRNA sequence database as a target. Primer sequences were redesigned and tested in the same way until optimal specificity was achieved. Novel 16S rRNA gene sequences were retrieved from GenBank with similarity searches with BLAST (2) and were analyzed for matches with the designed PCR primers.

PCR amplification, DNA analysis, and cloning. The following Ps-PCR primer set was developed: forward primer Ps-for (20-mer [5′-GGTCTGAGAGGATG ATCAGT-3′]) and reverse primer Ps-rev (18-mer [5′-TTAGCTCCACCTCGC GGC-3′]). According to the convention of the Oligonucleotide Probe Database (OPD [1]), the names of these primers were S-G-Psmn-0289-a-S-20 and S-G-Psmn-1258-a-A-18, respectively. The universal SSU primer set (11) included forward primer uni-for (17-mer [5′-TGCCAGCAGCCGCGGTA-3′]) and reverse primer uni-rev (18-mer [5′-GACGGGCGGTGTGTACAA-3′]). PCR cocktails for 100-μl reaction mixtures contained 1× reaction buffer (Boehringer Mannheim, Indianapolis, Ind.), 200 nM each deoxynucleoside (Boehringer Mannheim), 5 mg of bovine serum albumin (Sigma Co., St. Louis, Mo.) per ml, 200 nM each oligonucleotide primer (Center for Gene Research and Biotechnology, Oregon State University), and sample DNA in a volume of 95 μl. Approximately 10^5 cells or a corresponding amount of DNA from pure cultures or 0.5 μg of purified soil DNA was used as a template. PCR amplification was performed with a PTC-100 Thermal Cycler (M. J. Research, Inc., Watertown, Mass.) with a hot bonnet and was run with block temperature control. After initial denaturation for 5 min at 95°C, samples were maintained at 80°C to allow for hot start conditions and addition of 5 μl of enzyme solution containing 1 U of *Taq* DNA polymerase (Boehringer Mannheim) in 1× reaction buffer. PCR was performed with 30 or 40 temperature cycles under the standard high-stringency conditions listed in Table 2. The use of other annealing temperatures is indicated in the text. A 10-min final extension at 72°C was performed at the end of the cycling steps, and then samples were maintained at 4°C.

Five microliters of the PCR products was analyzed on 1% agarose gels (Gibco/BRL, Life Technologies Inc., Gaithersburg, Md.), and 5 μl was reserved for cloning. The remaining 90 μl of PCR products was mixed with 90 μl of precipitation solution (20% polyethylene glycol 8000 and 2.5 M NaCl [Sigma Co.]), and the mixture was incubated for 20 min at 37°C and microcentrifuged at a high speed for 15 min. DNA pellets were washed with 70% ethanol, air dried, and resuspended in 40 μl of 1× restriction enzyme buffer M with 2 U of restriction enzyme *Hae*III (Boehringer Mannheim). After overnight digestion at 37°C, restriction fragments were separated by gel electrophoresis with either 2% agarose (Gibco/BRL, Life Technologies, Inc.) or 4% MetaPhor (FMC BioProducts, Rockland, Maine). Molecular weight markers were either phage λ DNA digested with *Hin*dIII (Gibco/BRL, Life Technologies, Inc.) or phage ΦX174 DNA digested with *Hae*III (Boehringer Mannheim).

PCR products were cloned with the TA cloning kit (Invitrogen Co., San Diego, Calif.) according to the manufacturer's recommendation. For screening, small amounts of white colonies were added to a Ps-PCR with 30 cycles as described above. PCR product sizes and *Hae*III RFLP patterns were analyzed as described above.

The *Hae*III RFLP patterns of specific *Pseudomonas* PCR products were named by using the following system. Capital letters were used for patterns obtained from pure cultures or sequences retrieved from databases. Previously described RFLP patterns observed among cloned soil Ps-PCR products were named by the corresponding lowercase letters. Novel patterns observed among PCR product clones were labelled by lowercase letters of the end of the alphabet (w, x, y, and z).

DNA sequencing and sequence analyses. Plasmid DNA was prepared with Qiagen 100 plasmid midiprep columns according to the recommendations of the manufacturer (Qiagen, Inc., Chatsworth, Calif.). DNA sequencing was performed at the Center for Gene Research and Biotechnology, Oregon State University. For sequence determination of the cloned Ps-PCR products, a generally applicable sequencing strategy was developed. The sequences for entire cloned PCR products, approximately 990 bp in length, were determined by using four consecutive sequencing reactions on either DNA strand. In addition to the vector-encoded T7 and SP6 sequencing primer sites, forward primers 240-for (5′-TGCCAGCAGCCGCGGTA-3′), 520-for (5′-GATACCCTGGTAGTCCA CG-3′), and 800-for (5′-GTGCTGCATGGCTGTCGTC-3′) and reverse primers 280-rev (5′-GCTTTACGCCCAGTAATTC-3′), 520-rev (5′-CGTGGACTACC AGGGTATC-3′), and 800-rev (5′-GACGACAGCCATGCAGCAC-3′) were designed and used for sequencing. The names of these primers indicate the approximate locations and orientations within the Ps-PCR products. Sequence datum-derived *Hae*III RFLP fragmentation patterns were determined with MacDNASIS Pro version 3.2 software (Hitachi Software Engineering America, Ltd., San Bruno, Calif.). Similarity searches of the GenBank database were performed with BLAST (2), and those of the RDP database were performed with SIMILARITY_RANK and CHIMERA_CHECK (27). For phylogenetic analyses, novel sequences were manually aligned to the alignments retrieved from RDP by using the multiple alignment routine of MacDNASIS Pro version 3.2. Average linkage cluster analysis of aligned sequences for construction of phylogenetic trees was performed with Treecon version 1.15 (48). Pairwise distance values were determined by the two-parameter model (20), taking into account the fraction of positions differing by transition or transversion. Clustering was determined by unweighted pair group with mathematical averages (UPGMA) analysis of pairwise genetic distance values. Unrooted phylogenetic trees were deduced from 100 bootstrap samplings (9). Maximum parsimony and maximum likelihood analyses were performed by phylogenetic analysis using parsimony (PAUP) version 4.0.0d57 (43) on a Sparc 10 UNIX workstation. For heuristic-parsimony and maximum-likelihood runs, the maximum numbers of retained trees (Maxtrees parameters) were set to 2,000 and 100, respectively. All characters were treated as unordered, and an equal substitution rate was assumed. For each analysis, the strict consensus tree was determined, with the *Pseudomonas*-specific clone 6 (Ps-clone 6), defined as the outgroup sequence. The resulting trees were manually compared to the UPGMA tree determined with Treecon version 1.15 as described above.

Nucleotide sequence accession numbers. Nucleotide sequences have been deposited in the GenBank database under accession nos. AF006502 for Ps-clone 1, AF006503 for Ps-clone 3, AF006504 for Ps-clone 15, AF006505 for Ps-clone 4, AF006506 for Ps-clone 14, and AF006507 for Ps-clone 6.

RESULTS

Design and theoretical evaluation of Ps-PCR primers. The target regions for PCR primers Ps-for and Ps-rev were identified at locations 289 to 308 and 1258 to 1275, respectively, of the *P. aeruginosa* ATCC 10145 (type species and type strain) rRNA gene, which are homologous to locations 292 to 311 and

TABLE 1. Primer mismatch testing in RDP with the CHECK_PROBE algorithm

Sequence source and taxonomic description[a]	No. of primer/target mismatches[b]		RDP ribotype description[c]
	Ps-for	Ps-rev	
Beta (purple) bacteria			
Iodobacter fluviatile ATCC 33051	3	0	*Neisseria* group
Burkholderia solanacearum ATCC 11696	1	0	*Burkholderia* subgroup I
Burkholderia pickettii ATCC 27512	1	0	*Burkholderia* subgroup I
Spirillum voluntans ATCC 19554	0	3	*Spirillum voluntans* group
Azoarcus sp. strain S5b2	1	0	*Rhodocyclus* group
Azoarcus indigens VB32	1	0	*Rhodocyclus* group
Gamma (purple) bacteria			
Ectothiorhodospira halochloris ATCC 35916	>3	0	*Ectothiorhodospira* group
Coxiella burnetii Q177	1	0	*Legionella* group
Methylococcus capsulatus bath	2	0	*Methylomonas* group
Oceanospirillum minutulum ATCC 19193	0	3	*Halomonas* group
Moraxella osloensis	0	NS	*Pseudomonas* and relatives group
Moraxella osloensis SCB111	1	0	*Pseudomonas* and relatives group
Pseudomonas aeruginosa ATCC 10145	0	2	*Pseudomonas* and relatives group[d]
Pseudomonas aeruginosa ATCC 25330	1	0	*Pseudomonas* and relatives group[d]
Pseudomonas aeruginosa NF13	0	NS	*Pseudomonas* and relatives group[d]
Flavobacterium lutescens ATCC 27951	0	0	*Pseudomonas* and relatives group[d]
Pseudomonas mendocina ATCC 25411	0	0	*Pseudomonas* and relatives group[d]
Pseudomonas flavescens B62 NCPPC 3063	0	0	*Pseudomonas* and relatives group[d]
Pseudomonas putida PaW1 isolate mt-2	0	0	*Pseudomonas* and relatives group[d]
Pseudomonas syringae A501	0	0	*Pseudomonas* and relatives group[d]
Pseudomonas fluorescens ATCC 13525	0	0	*Pseudomonas* and relatives group[d]
Azospirillum sp. strain DSM 1727	0	0	*Pseudomonas* and relatives group[d]
Pseudomonas syringae 31R-1	0	0	*Pseudomonas* and relatives group[d]
Pseudomonas syringae env. FIE8	NS	0	*Pseudomonas* and relatives group[d]
Pseudomonas fluorescens MS 1650	0	0	*Pseudomonas* and relatives group[d]
Shewanella putrefaciens ATCC 8071	3	0	*Alteromonas* group
Delta (purple) Bacteria			
Bdellovibrio bacteriovorus ATCC 43826	0	2	*Bdellovibrio bacteriovorus* group
Desulfobacter sp. strain DSM 2057	0	>3	*Desulfobacter* subgroup
Nannocystis exedens ATCC 25963	0	>3	*Myxobacteria* subgroup
Epsilon (purple) bacteria			
Bacteroides ureolyticus ATCC 33387	0	>3	*Campylobacter* and relatives group
Bacteroides gracilis ATCC 33236	0	2	*Campylobacter* and relatives group
Campylobacter (13 species and 31 sequences)	0	>3	*Campylobacter* and relatives group
Fuscobacteria and relatives			
Fusobacterium periodonticum ATCC 33693	>3	0	*Fuscobacteria* and relatives group
Gram-positive bacteria			
Haloanaerobium salsugo VS-752	0	>3	Anaerobic halophiles subgroup
Haloanaerobium praevalens ATCC 33744	0	3	Anaerobic halophiles subgroup
Clostridium bifermentans NCIMB 10716	>3	0	*Clostridium lituseburense* group
Clostridium ghonii ATCC 25757	>3	0	*Clostridium lituseburense* group
Listeria monocytogenes ATCC 35152	>3	0	*Listeria-Brochothrix* group

[a] CHECK_PROBE searches of the RDP prokaryotic SSU database. Sequences perfectly matching with at least one primer were extracted and are listed in phylogenetic order.

[b] The CHECK_PROBE searches allowed as many as three mismatches. The numbers of mismatches observed for the Ps-for and Ps-rev primer targets are listed for each sequence. NS, no primer target site sequence was available; >3, primer target sites exceeding the search cutoff of three mismatches.

[c] Ribotype classifications of the listed sequences as defined by RDP.

[d] The 13 sequences of the *Pseudomonas* subgroup in RDP.

1263 to 1280 in the *Escherichia coli* 16S rRNA gene, respectively. Theoretical hybridization targets for each primer were determined by extensively searching the 2,849 prokaryotic SSU rRNA gene sequences in RDP (Table 1). Ps-for (the forward primer) displayed a perfect match with 11 of 12 available *Pseudomonas* sequences and 12 of the 2,836 non-*Pseudomonas* sequences. Ps-rev (the reverse primer) perfectly matched 11 of 12 *Pseudomonas* sequences and 44 non-*Pseudomonas* sequences. For the non-*Pseudomonas* sequence matches, these values represented 0.4 and 1.5%, respectively, of the entire RDP database. In combination, the two primers exhibited a high degree of selectivity for the RDP *Pseudomonas* subgroup. The only exceptions were two *P. aeruginosa* sequences (cultures ATCC 10145 and ATCC 25330), each showing mismatches in a different primer (Table 1). The same analysis revealed that all available non-*Pseudomonas* sequences in the database dis-

TABLE 2. PCR temperature cycling conditions on an MJR PTC-100 operated with block control

Method	Sample type	Temp (°C)[a] under the following conditions and incubation times[b]:					
		Denaturation		Annealing		Extension	
		11 s	15 s	8 s	1 min	10 s	1 min
Ps-PCR[c]	Cultures	94	92	66	68	74	72
Ps-PCR[c]	Soil DNA	94	92	63	65	74	72
uni-PCR[d]	Cultures	94	92	60	62	74	72

[a] Target block temperature for which the temperature cycler was programmed.
[b] Time period for which the block of the temperature cycler was maintained at the given target temperature.
[c] PCR performed with primers Ps-for and Ps-rev.
[d] PCR performed with the universal SSU primers uni-for and uni-rev.

played mismatches for at least one primer, confirming the *Pseudomonas* selectivity of these primer regions and supporting the feasibility of a 16S rRNA-based Ps-PCR protocol. Amplification may also occur with some non-*Pseudomonas* sequences of the beta and gamma proteobacterial subdivisions, which display only one mismatch in one of the primers (Table 1) or possibly with sequences not documented in RDP. For further testing of the Ps-PCR primers, 51 recently published *Pseudomonas* 16S rRNA sequences encoding the whole PCR target sequence were identified in the GenBank database, retrieved, and tested for mismatches (for information on these sequences and references, see Fig. 4). Five of these sequences, i.e., 3 of 17 *P. stutzeri* (ATCC 11607, LS 401, and SP 1402) sequences, the *P. tolaasii* (ATCC 33618) sequence, and 1 of 2 *Chryseomonas luteola* (IAM 13000) sequences, were found to display single mismatches in one primer region. In summary, of 64 tested *Pseudomonas* 16S rRNA sequences retrieved from the RDP and GenBank databases, only 8 (12.5%) displayed mismatches with the primers, whereas none of 2,836 tested non-*Pseudomonas* sequences displayed a perfect match for both primers.

Experimental testing of the Ps-PCR protocol on pure cultures. The specificities of the Ps-PCR primers on 64 pure cultures were experimentally tested (Table 3), with PCR performed at annealing temperatures of 62, 65, 68, or 72°C. The cultures analyzed included 18 *Pseudomonas* and 11 non-*Pseudomonas* cultures obtained from culture collections and 32 *Pseudomonas* and 3 non-*Pseudomonas* soil isolates identified with the Biolog system. The analyses revealed that all 50 *Pseudomonas* (sensu stricto) cultures tested positive by the Ps-PCR protocol, while the 14 non-*Pseudomonas* cultures tested negative. All cultures tested positive by the universal-PCR protocol for SSU rRNA genes (Table 3). The 16S rDNA sequence of *Burkholderia pickettii* (ATCC 27511) displayed 1 mismatch with the forward Ps-PCR primer (Table 1) and was the only non-*Pseudomonas* sequence tested that required the high annealing temperature of 68°C to prevent its amplification. All *Pseudomonas* cultures tested positive at this high annealing temperature, including *P. aeruginosa* (ATCC 10145), the published sequence of which displayed two mismatches with the reverse primer (Table 1). No amplification was observed when annealing was performed at 72°C (data not shown). Figure 1 shows the 16S rRNA gene PCR analyses of 18 pure cultures with the universal SSU primer set (Fig. 1a) or the Ps-PCR primer set (Fig. 1b). PCR products (Fig. 1a1 and 1b1) and corresponding *Hae*III RFLPs (Fig. 1a2 and 1b2) are shown. The three RFLP patterns obtained from Ps-PCR products were pattern A (Fig. 1b2, lanes 3, 4, 7 to 12, and 14 to 17), pattern B (Fig. 1b2, lane 13), and pattern C (Fig. 1b2, lane 18).

TABLE 3. Results of experimental specificity testing of pure cultures with the Ps-PCR primers

Culture description[a]	*Hae*III RFLP pattern	
	Ps-PCR[b]	Universal PCR[c]
Culture collections		
P. putida type B ATCC 17527*	A	Psm.A
P. putida ATCC 11172	A	Psm.A
P. aeruginosa ATCC 10145	A	Psm.A
P. syringae pv. syringae Biolog 7350*	A	Psm.A
P. syringae pv. syringae EPA 22A-93	A	Psm.A
P. syringae pv. tomato EPA PT-23	A	Psm.A
P. syringae pv. phaseolica EPA JA-019	A	Psm.A
P. cichorii ATCC 10857*	A	Psm.A
P. taetrolens Biolog 8570*	A	Psm.A
P. synxantha ATCC 9890*	A	Psm.A
P. fragi ATCC 4973*	A	Psm.A
P. resinovorans ATCC 14235*	A	Psm.A
P. mendocina ATCC 25411*	A	Psm.A
P. agarici ATCC 25941*	B	Psm.B
P. fulva ATCC 31418*	A	Psm.A
P. stutzeri ATCC 17588*	A	Psm.A
P. alcaligenes B ATCC 14909*	A	Psm.A
P. aureofaciens EPA 604	A	Psm.A
Burkholderia pickettii ATCC 27511	−	BuhI.A
Burkholderia andropogonis ATCC 23061*	−	BuhII.A
Burkholderia cepacia ATCC 25416	−	BuhII.A
Comamonas testosteroni ATCC 11996	−	Com.A
Acidovorax facilis ATCC 11228	−	Acv.A
Hydrogenophaga flava ATCC 33667	−	Hyp.A
Brevundimonas vesicularis ATCC 11426	−	Brm.A
Stenotrophomonas maltophila ATCC 13637	−	Stm.A
P. floridana ATCC 25616*	−	"Psm".A
Pseudomonas species-like group II Biolog 1574*	−	"Psm".A
P. boreopolis ATCC 3242*	−	"Psm".B
Biolog-identified environmental isolates		
P. putida A1 isolates E8P3D2, E12P3C1, F2P4B3, and 77ys	A	Psm.A
P. putida B1 isolates F2C1B1, BMD B2, and BMD B7	A	Psm.A
P. fluorescens type A isolate E2P5B1	A	Psm.A
P. fluorescens type B isolates 75csr, 137ymr, 182ysr, and 228csr	A	Psm.A
P. fluorescens type C isolate F4C3D3	A	Psm.A
P. fluorescens type G isolates E2P5A3, F2C1B3, 27cm, 129,ymr, 7A, and 2A	A	Psm.A
P. marginalis isolates A5P4C3 and 18B	C	Psm.A
P. corrugata isolates E8C1D1 and 104ysr	A	Psm.A
P. viridilivida isolates E2P4B3 and E2P4A1	A	Psm.A
P. aurantiaca isolates RES A5 and FB49H	A	Psm.A
P. viridiflava isolate RES A7	A	Psm.A
P. fragi isolate 7C	A	Psm.A
P. fuscovaginae isolate 7B	A	Psm.A
Pseudomonas sp. isolate RES A4	A	Psm.A
Pseudomonas sp. isolate BMD B36	A	Psm.A
Acinetobacter sp. isolate 78 cs	−	Acb.A
Acinetobacter calcoacitices isolate FA18C	−	Acb.A
Alcaligenes xylosoxidans isolate FA30G	−	Alc.A

[a] Fifty *Pseudomonas* and 14 non-*Pseudomonas* cultures used to test the specificity of the Ps-PCR protocol. *, culture DNA sample kindly provided by Biolog Inc.
[b] The *Hae*III RFLP patterns of Ps-PCR products. The three detected patterns (as shown in Fig. 1b2) were labelled A, B, and C. −, cultures that did not yield an amplification product by the Ps-PCR protocol with 30 cycles at an annealing temperature of 68°C.
[c] The *Hae*III RFLP patterns of PCR products obtained with the universal SSU rRNA gene primer set. Patterns (as shown in Fig. 1a2) were labelled with a genus abbreviation followed by a capital letter. Psm, *Pseudomonas*; BuhI, *Burkholderia* type I; BuhII, *Burkholderia* type II; Com, *Comamonas*; Hyp, *Hydrogenophaga*; Brm, *Brevundimonas*; Stm, *Stenotrophomonas*; Acb, *Acinetobacter*; Alc, *Alcaligenes*; quotation marks, non-*Pseudomonas* (sensu stricto) cultures that are still named *Pseudomonas*.

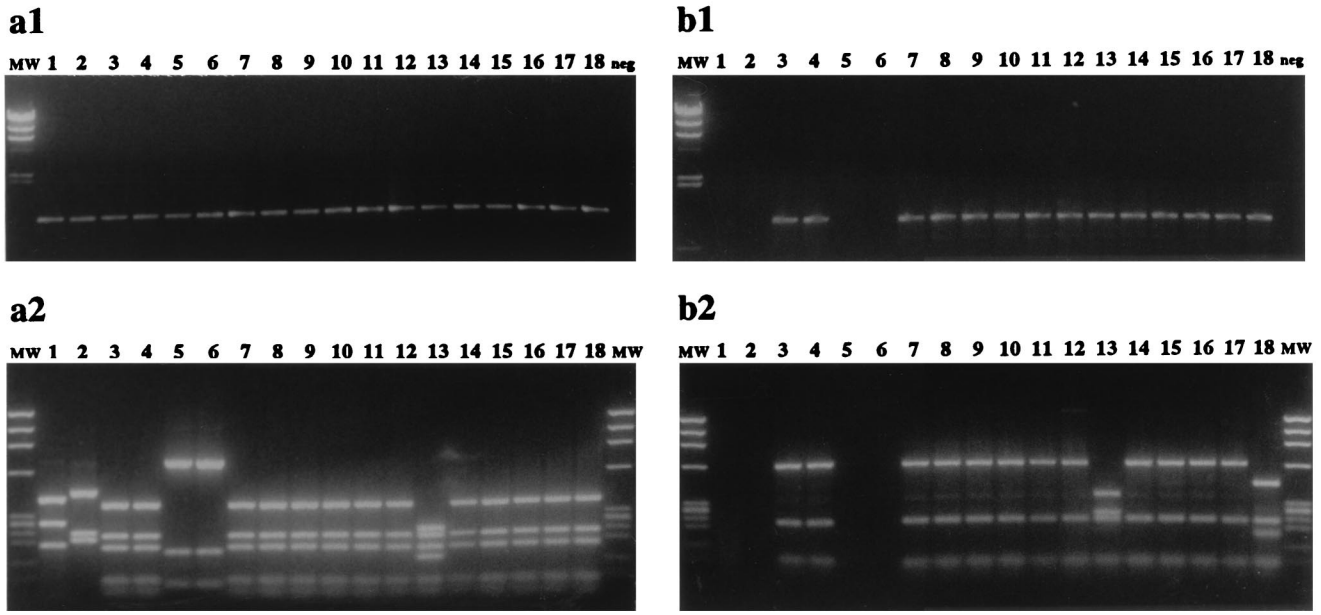

FIG. 1. PCR amplification and *Hae*III RFLP patterns of pure cultures. PCR amplification (30 cycles) of 16S DNA coding for rRNA of pure cultures was performed either with a universal SSU primer set (a) or with the Ps-PCR primer set (b). (a1 and b1) Amplification products from cultures (lanes 1 to 18) and a negative control (neg) on 1% agarose gels. The molecular weight marker (MW) was λ *Hin*dIII. (a2 and b2) *Hae*III RFLP patterns on 2% agarose gels. The molecular weight marker (MW) was ΦX174 *Hae*III. The 18 cultures were "*Pseudomonas*" *boreopolis* (ATCC 3242) (lane 1), *Burkholderia andropogonis* (ATCC 23061) (lane 2), *P. cichorii* (ATCC 10857) (lane 3), *P. syringae* pv. syringae (Biolog 7350) (lane 4), "*P.*" *floridana* (ATCC 25616) (lane 5), "*Pseudomonas*" sp.-like group II (Biolog 1574) (lane 6), *P. taetrolens* (Biolog 8570) (lane 7), *P. putida* biotype B (ATCC 17527) (lane 8), *P. synxantha* (ATCC 9890) (lane 9), *P. fragi* (ATCC 4973) (lane 10), *P. recinovorans* (ATCC 14235) (lane 11), *P. mendocina* (ATCC 25411) (lane 12), *P. agarici* (ATCC 25941) (lane 13), *P. fulva* (ATCC 31418) (lane 14), *P. stutzeri* (ATCC 17588) (lane 15), *P. alcaligenes* (ATCC 14909) (lane 16), *P. fluorescens* biotype G (isolate 27cm) (lane 17), and *P. marginalis* (isolate A5P4C3) (lane 18). The *Pseudomonas* genus names that are in quotation marks are not *Pseudomonas* (sensu stricto) cultures.)

These RFLP patterns represented all of the patterns observed for the pure cultures tested in this study. The *Hae*III RFLP patterns shown for the universal SSU primer set (Fig. 1a2) represented 5 of 13 described in this study (Table 3) and were labelled "Psm".A (lane 1), BurII.A (lane 2), Psm.A (lanes 3, 4, 7 to 12, and 14 to 18), "Psm".B (lanes 5 and 6), and Psm.B (lane 13). The quotation marks in Psm indicate that these patterns did not represent *Pseudomonas* (sensu stricto) cultures. In summary, the Ps-PCR protocol was suitable for specific amplification of 16S rRNA *Pseudomonas* genes, and *Hae*III RFLP analysis of Ps-PCR products experimentally confirmed the specificity of the Ps-PCR method for target organisms at 100%.

Ps-PCR analysis of total DNA from an agricultural soil sample. To further evaluate the specificity of the Ps-PCR protocol, purified DNA extracts from an agricultural soil sample were used as a complex source for 16S rRNA gene amplification targets of culturable and nonculturable microorganisms. Two soil DNA preparations extracted from 0.5 g of subsamples of a 100-g soil sample were used for amplification, with annealing temperatures of 62, 65, and 68°C. Amplification products were obtained only for the 62 and 65°C annealing temperatures (Fig. 2a). *Hae*III RFLP analysis of the Ps-PCR amplification products revealed small variations in relative band intensities between subsamples. These variations were reproducible and stable at the two annealing temperatures (Fig. 2b). The patterns were composed of seven prominent bands, suggesting a relatively low complexity of the main amplification target in this soil. Some of the bands migrated at molecular weights identical to those for the RFLP pattern A observed for the majority of pure *Pseudomonas* cultures (arrows in Fig. 2b; Fig. 1b2, lanes 3, 4, 7 to 12, and 14 to 17), suggesting that *Pseudomonas* 16S rRNA sequences represented a highly enriched component in the amplification product.

Cloning of Ps-PCR products from agricultural soil DNA. To identify the components present in the soil Ps-PCR-*Hae*III RFLP fingerprint as illustrated in Fig. 2b, Ps-PCR products (62°C annealing) were cloned. The library was screened by the Ps-PCR protocol in combination with *Hae*III RFLP analysis. Of 40 clones screened, 36 yielded positive Ps-PCR results (Ps-

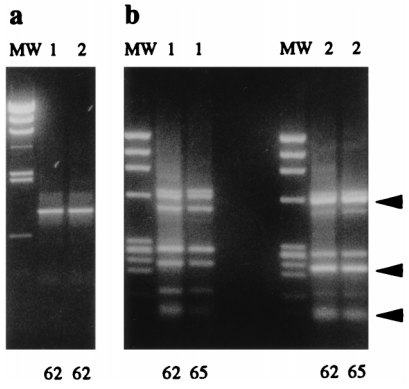

FIG. 2. Ps-PCR products and *Hae*III RFLP patterns obtained from bulk DNA purified from an agricultural soil sample. PCR amplification (40 cycles) was performed with DNA from two soil subsamples (1 and 2) with annealing temperatures of either 62 or 65°C (62 or 65, respectively). (a) PCR products in a 1% agarose gel (only products of the 62°C annealing are shown). The molecular weight marker was λ *Hin*dIII. (b) *Hae*III RFLP patterns in a 2% agarose gel. The molecular weight marker (MW) was ΦX174 *Hae*III. Arrows, positions of RFLP pattern A shown in Fig. 1b2 (lanes 3, 4, 7 to 12, and 14 to 17).

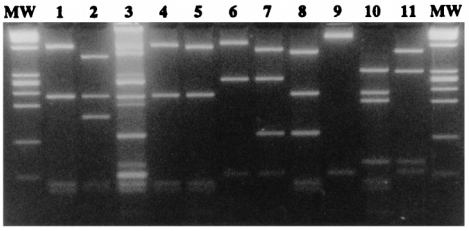

FIG. 3. *Hae*III RFLP patterns of Ps-PCR products from pure *Pseudomonas* cultures, total soil DNA, and Ps-clones. Lanes 1 and 2, patterns A and C obtained from the field isolates *P. fluorescens* and *P. marginalis*, respectively; lane 3, high-resolution analysis of the soil DNA fingerprint shown in Fig. 2b (lane 1; 62°C annealing). The eight RFLP patterns (lanes 4 to 11) found among the 36 Ps-clones were pattern a (Ps-clone 3 [lane 4]), pattern d (Ps-clone 15 [lane 5]), pattern j (Ps-clone 1 [lane 6]), pattern w (Ps-clone 4 [lane 7]), pattern e (Ps-clone 14 [lane 8]), pattern x (Ps-clone 13 [lane 9]), pattern y (Ps-clone 2 [lane 10]), and pattern z (Ps-clone 6 [lane 11]). MetaPhor gel (4%) with molecular weight marker (MW) ΦX174 *Hae*III was used.

clones). RFLP analysis revealed eight different fragmentation paterns (Fig. 3, lanes 4 to 11). Pattern a (Fig. 3, lane 4) occurred 20 times (55.6%), was identical to pattern A observed for the majority of pure *Pseudomonas* cultures (Fig. 1b2, lanes 3, 4, 7 to 12, and 14 to 17), and is again shown in Fig. 3, lanes 1 and 4. Pattern w (Fig. 3, lane 7) occurred 10 times (27.7%). The other six patterns (d, e, j, x, y, and z; Fig. 3, lanes 5, 6, and 8 to 11) were all represented once (2.7% each) among the 36 Ps-clones. Gel electrophoresis through 4% MetaPhor revealed comigration of the eight identified patterns (Fig. 3, lanes 4 to 11) with the banding pattern observed in the complex soil DNA fingerprint (Fig. 3, lane 3). This finding, together with the observed frequency of the patterns, indicated that the 36 Ps-clones comprised the majority of Ps-PCR products amplified from the analyzed soil DNA.

Sequence analysis of Ps-PCR products from the agricultural soil DNA. Complete sequences were determined for eight Ps-clones representing each of the eight RFLP patterns observed, followed by extensive sequence analyses. Similarity searches in the RDP and GenBank databases revealed that the sequences were of proteobacterial and dominantly *Pseudomonas* origin. CHIMERA_CHECK analysis in RDP suggested that Ps-clones 2 and 13 (patterns y and x) represented chimeric PCR products of sequences derived from the *Oceanospirillum* assemblage and *Xanthomonas* group or *Nitrosomonas* group and *Pseudomonas* subgroup genes, respectively (data not shown). SIMILARITY_RANK analyses indicated that Ps-clones 1, 3, and 15 (patterns j, a, and d, respectively) were *Pseudomonas* sequences and that Ps-clone 6 (pattern z) was derived from a microorganism closely related to the *Nitrosomonas* or *Rhodocyclus* group. The results obtained for Ps-clones 4 and 14 (patterns w and e, respectively) were not conclusive, since they indicated the presence of closely related sequences from the *Oceanospirillum* group and *Pseudomonas* subgroup or *Legionella* and *Pseudomonas* subgroups, respectively. The findings that *Hae*III RFLP pattern w, represented by Ps-clone 4, occurred 10 times (27.7%) among the analyzed Ps-clones and that its banding pattern was found by independent PCR suggested that it did not represent a chimeric product. The phylogenetic analyses presented in the following section confirmed and further clarified these initial findings.

In order to evaluate a phylogenetic analysis approach, the 11 full-length *Pseudomonas* sequences and 4 selected closely related gamma proteobacterial outgroup sequences that encoded the entire Ps-PCR target region were obtained in the form of the 1,007-bp-long RDP alignment (Fig. 4, underlined names). Cluster analysis of this data set with 100 bootstrap samplings resulted in a phylogenetic tree consistent with the RDP phylogenetic data. The *Pseudomonas* sequences formed a distinct cluster that was joined by a branch of the closely related outgroup sequences (data not shown [but see Fig. 4]). This finding indicated that the Ps-PCR target area contained sufficient information to obtain phylogenetic results consistent with published data.

The 51 new *Pseudomonas* sequences and 4 new closely related outgroup sequences retrieved from GenBank and the 6 confirmed Ps-clone sequences were aligned with the 11 *Pseudomonas* and 4 non-*Pseudomonas* sequences, obtained as a 1,007-bp alignment from RDP. To align all 76 sequences, some adjustments of the RDP alignment were required, resulting in a final alignment length of 1,018 bp (data not shown). This data set was subjected to cluster analysis with 100 bootstrap samplings, resulting in the unrooted phylogenetic tree shown in Fig. 4. The 11 RDP *Pseudomonas* 16S rDNA sequences (9 from named *Pseudomonas* species plus those originally identified as *Flavobacterium lutescens* and *Azospirillum* isolates) did cluster with 52 related sequences, 47 of which were derived from named *Pseudomonas* strains. The 5 differently named sequences were from *C. luteola* (ATCC 43330 and IAM 13000), *Flavimonas oryzihabitans* (IAM 1568), and Ps-clones 3 and 15. This cluster of 63 sequences formed three main branches (I, II, and III in Fig. 4). Each of these branches was characterized by the specific occurrence of *Pseudomonas* (sensu stricto) species, i.e., *P. putida* and *P. stutzeri* in branch I, *P. aeruginosa* in branch II, and *P. fluorescens* and *P. syringae* in branch III. Ps-clones 3 and 15 localized in different subclusters of branch III. At the same branching point where these main branches originated, a *P. oleovorans* branch (one sequence) and a *C. luteola* branch (two sequences) diverged. Six sequences clustered in three branches between these 63 *Pseudomonas* 16S rDNA sequences and the sequence of SCB11, the outgroup 16S rDNA sequence most closely related to the genus *Pseudomonas* in RDP. These three branches were formed by Ps-clone 1 together with the undefined *Pseudomonas* isolate CRE 12; two Lake isolates, 45E3 and 52N3; and Ps-clones 4 and 14. All 69 sequences were located within the target area of the PS-PCR protocol (Fig. 4, open arrow) and formed a distinct cluster with a bootstrap value of 99%. Extensive cluster analysis of the Ps-clone 6 sequence and RDP proteobacterial sequences revealed that it clustered between the *Gallionella* assemblage (RDP group 2.13.2.5) and *Nitrosomonas* group (RDP group 2.13.2.6) of the beta proteobacterial subdivision (data not shown). Analyses of the same data set by the maximum-parsimony and maximum-likelihood methods revealed similar results, with two main differences compared to those for UPGMA analysis (data not shown). All methods identified the three clusters (Fig. 4); however, the parsimony and maximum-likelihood analyses identified branch III as a subgroup of branch I. The outgroup sequences also clustered, in agreement with the UPGMA analysis. The second difference involved the nine sequences within the target area of the Ps-PCR (Fig. 4, open arrow) but not located in branches I, II, and III. Parsimony analysis identified exactly the same target area as the UPGMA analysis but located Ps-clones 4 and 14 in branch III and Ps-clone 1 in branch I. Maximum-likelihood analysis located *P. oleovorans* (IAM 1508), the two lake isolates 45E3 and 52N3, and the *Pseudomonas* sp. isolate CRE 12 in the outgroup cluster. The two *C. luteola* (ATCC 43330 and IAM 13000) sequences clustered in branch I, and Ps-clone 1, 4, and 14 sequences clustered in branch III. In summary, all of these analyses revealed that the five Ps-clone sequences 1, 3, 4, 14, and 15, representing pat-

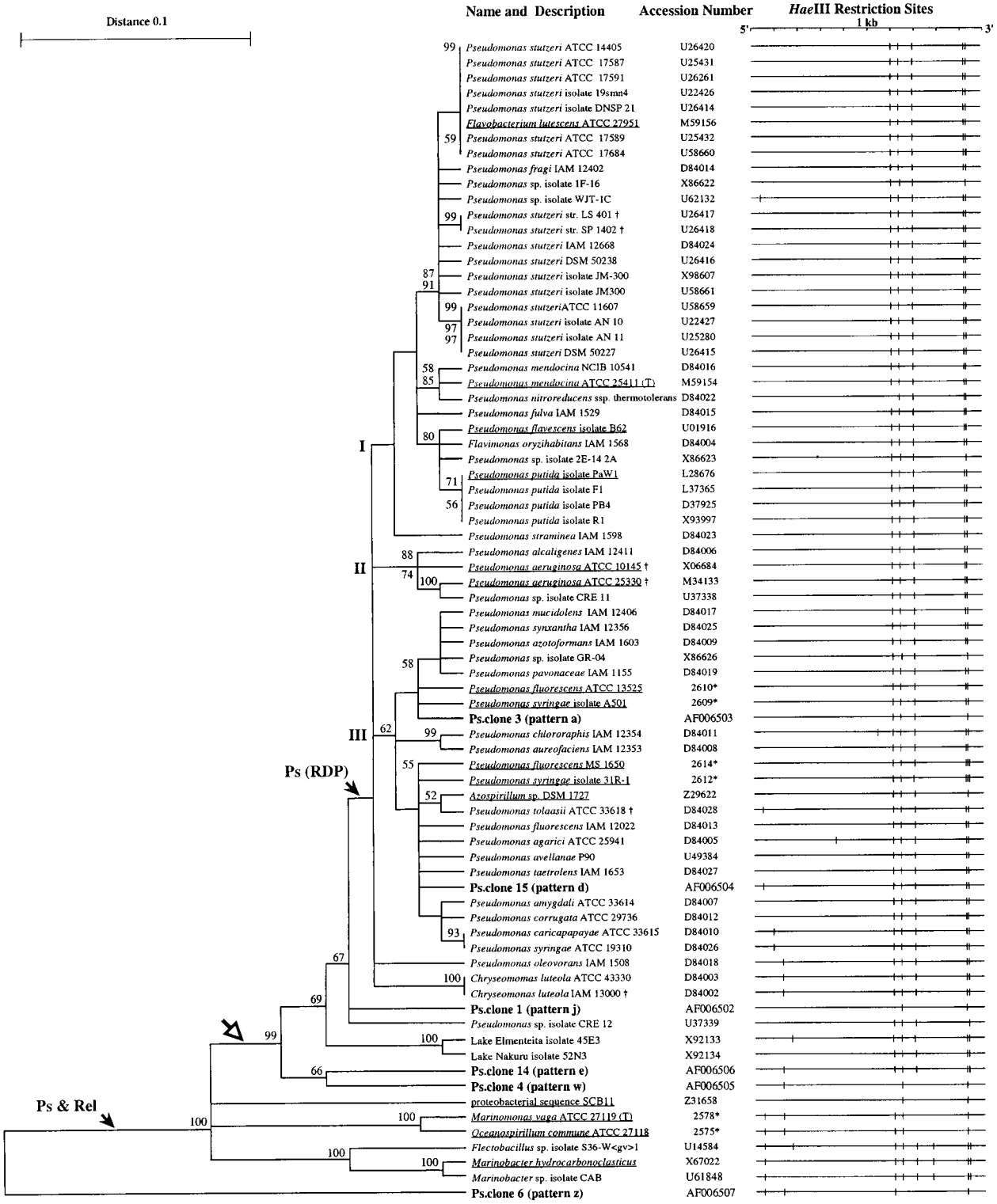

FIG. 4. Phylogenetic analyses of the 6 confirmed Ps-clone sequences (boldface letters) and 70 control sequences from RDP (underlined) and GenBank. The unrooted phylogenetic tree is the result of UPGMA cluster analysis with 100 bootstrap samplings. Bootstrap values larger than 50 are shown. The arrow marked Ps & Rel indicates the branch closely corresponding to the group *Pseudomonas* and relatives in RDP. The arrow marked Ps (RDP) indicates the cluster containing the *Pseudomonas* subgroup from RDP. The subbranches labelled I, II, and III are characterized by the specific occurrence of marker species such as *P. putida* in I, *P. aeruginosa* in II, and *P. fluorescens* and *P. syringae* in III. The open arrow indicates the designed target area of the Ps-PCR protocol. For reference, the GenBank accession or RDP number (*) is listed. †, sequences within the Ps-PCR target area which displayed mismatches with one of the primers. To demonstrate the diagnostic value of *Hae*III RFLP patterns for a majority of *Pseudomonas* identifications, the *Hae*III restriction maps of the Ps-PCR target areas are shown.

terns j, a, w, e, and d, respectively, and 91.7% of the Ps-clones, clustered within the target area of the Ps-PCR protocol.

DISCUSSION

This study was conducted to develop a 16S rDNA-based PCR protocol for the detection and identification of members of the genus *Pseudomonas* (sensu stricto). The Ps-PCR primers were subjected to theoretical testing with database information and experimental testing on pure cultures and bulk DNA from an agricultural soil sample.

Database searches for 16S rDNA similarities revealed sequences related to *Pseudomonas* (sensu stricto) that derived from cultures originally identified by others as belonging to different genera. *Pseudomonas* classification has attracted much attention in the recent past, and extensive reclassification has been performed (5, 19, 34, 35). However, there still exist uncertainties (Table 1 and Fig. 4) (27). The Ps-PCR protocol may offer a rapid diagnostic tool for genus identification, which was strongly supported by its experimentally confirmed selectivity and the RFLP patterns obtained for *Pseudomonas* species (Fig. 1 and Table 3).

The 16S rRNA sequences retrieved from RDP and GenBank indicated that about 10% of the *Pseudomonas* sequences may display mismatches with the Ps-PCR primers (Table 1 and Fig. 4). Experimental evaluation of the PCR approach with pure cultures (Fig. 1 and Table 3) showed that 16S rRNA genes from a variety of *Pseudomonas* species were specifically amplified by the Ps-PCR. The test included *P. aeruginosa* (ATCC 10145), the published sequence of which displayed two mismatches with the reverse primer (44). This positive result under the high-stringency annealing condition of 68°C indicated a perfect match of the primers, since at the same temperature no product was obtained for *B. pickettii* (ATCC 27512; also referred to as *Ralstonia pickettii* [51]), which has only one mismatch in the forward primer (Table 1). At 72°C annealing, no amplification was observed for any of the samples, indicating the high stringency of the PCR conditions. Database information and available analysis tools (27) were very helpful in the process of primer design, but some caution should be used when interpreting the retrieved information. PCR or cloning artifacts and sequencing ambiguities may account for some of these sequence differences. It cannot be ruled out that some *Pseudomonas* isolates may have primer target sequences diverging from those described here; however, this was not observed among the 50 pure *Pseudomonas* cultures.

Stringency evaluation similar to that performed for pure cultures and discussed above was performed for the bulk soil DNA. The results revealed that for such samples, the annealing temperature needed to be lowered to 65°C (Fig. 3). This lower annealing temperature for the soil DNA may be explained by residual soil contaminants slightly altering the conditions for optimal PCR amplification (50). Analysis of *B. pickettii* indicated that an annealing temperature decrease by 3°C below the highest stringency may allow for amplification of sequences with single mismatches. Since the database analysis indicated that some *Pseudomonas* sp. strains may exhibit single mismatches and that no significant changes were observed between soil DNA fingerprints with annealing temperatures of 62 and 65°C (Fig. 2), the product of a PCR at 62°C annealing was cloned and analyzed in detail. Among 36 Ps-clones, eight different RFLP patterns were observed. Three patterns that represented only 8.3% of the clones were derived from nontarget sequences. Two were from chimeric PCR products which are a known possible artifact of PCR (26, 30), and one was a beta proteobacterial nontarget sequence. Thus, the reduced annealing temperature did not result in a high rate of nontarget sequence amplification. However, sequences with one mismatch may be more frequent in other samples, and it is recommended that the optimal annealing temperature be evaluated with preliminary DNA fingerprint analysis before cloning Ps-PCR amplification products.

Among the 50 *Pseudomonas* cultures that represented 21 species, 3 different *Hae*III RFLP patterns were detected (Fig. 2; Table 3). Pattern A was found in 47 cultures (94%). The prominent occurrence of this pattern was confirmed by analysis of database information (Fig. 4). Among 63 *Pseudomonas* sequences in the databases, 52 (82.5%) displayed the *Hae*III RFLP pattern A. Among the *Pseudomonas* cultures, pattern B was found once (2%) in *P. agarici* and was confirmed in GenBank with one occurrence (Table 3 and Fig. 4). Pattern C was derived from two of the tested cultures identified as *P. marginalis* with the Biolog system (Table 3). Six additional *Pseudomonas* sp. *Hae*III RFLP patterns were identified in GenBank and were labelled patterns D through I (Fig. 4). The low variability of *Pseudomonas* 16S RFLP patterns has been reported earlier (25) and was in agreement with the database analysis shown in Fig. 4. The strong relatednes of the *Hae*III RFLP patterns of Ps-PCR products may be used as a diagnostic criterion to assist with *Pseudomonas* identification.

Phylogenetic analyses revealed that the majority of *Pseudomonas* sequences clustered in three main branches (Fig. 4) characterized by *P. putida* and *P. stutzeri* (branch I), *P. aeruginosa* (branch II), and *P. fluorescens* and *P. syringae* (branch III). High similarity between the group of *Pseudomonas* and relatives in this study and the one of RDP was found, and a possible cluster of the genus *Pseudomonas* (sensu stricto) was identified (Fig. 4). This potential *Pseudomonas* branch formed the target area of the Ps-PCR protocol, included five of the six confirmed RFLP patterns found among the Ps-clones, and was largely supported by the three phylogenetic analyses performed in this study. In summary, five sequences (Ps-clones 1, 3, 4, 14, and 15) representing 91.7% of the Ps-clones clustered in the *Pseudomonas* target area and strongly supported the predicted high *Pseudomonas* selectivity of the Ps-PCR primer pair.

The Ps-PCR protocol in conjunction with RFLP analysis, sequence determination, and phylogenetic analyses may be used to compare *Pseudomonas* population structures from a variety of ecosystems and provide further insight about the occurrence, potential roles, and possible unidentified subgroups of this genus in different ecosystems. In addition, it may represent a rapid assay for confirmation of members of the genus *Pseudomonas* (sensu stricto).

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