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Oligonucleotide Primers Designed To Differentiate Pathogenic Pseudomonads on the Basis of the Sequencing of Genes Coding for 16S-23S rRNA Internal Transcribed Spacers

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Universal primers targeting conserved sequences flanking the 3′ end of the 16S and the 5′ end of the 23S rRNA genes (rDNAs) were used to amplify the 16S-23S rDNA internal transcribed spacers (ITS) from eight species of pseudomonads which have been associated with human infections. Amplicons from reference strains of *Pseudomonas aeruginosa*, *Pseudomonas cepacia*, *Pseudomonas gladioli*, *Pseudomonas mallei*, *Pseudomonas mendocina*, *Pseudomonas pickettii*, *Pseudomonas pseudomallei*, and *Xanthomonas maltophilia* were cloned from each species, and sequence analysis revealed a total of 19 distinct ITS regions, each defining a unique sequevar with ITS sizes ranging from 394 (*P. cepacia*) to 641 (*P. pseudomallei*) bp. Five distinct ITS sequevars in *P. cepacia*, four in *P. mendocina*, three in *P. aeruginosa*, two each in *P. gladioli* and *P. pseudomallei*, and one each in *P. mallei*, *P. pickettii*, and *X. maltophilia* were identified. With the exception of one *P. cepacia* ITS, all ITS regions contained potential tRNA sequences for isoleucine and/or alanine. On the basis of these ITS sequence data, species-specific oligonucleotide primers were designed to differentiate *P. aeruginosa*, *P. cepacia*, and *P. pickettii*. The specificities of these primers were investigated by testing 220 clinical isolates, including 101 strains of *P. aeruginosa*, 103 strains of *P. cepacia*, and 16 strains of *P. pickettii*, in addition to 24 American Type Culture Collection (ATCC) *Pseudomonas* strains. The results showed that single primer pairs directed at particular ITSs were capable of specifically identifying the ATCC reference strains and all of the clinical isolates of *P. aeruginosa* and *P. pickettii*, but this was not the case with several ITS-based primer pairs tested for *P. cepacia*. This pathogen, on the other hand, could be specifically identified by primer pairs directed against the 23S rDNA.

Amplification-based methods are having a significant impact on the identification and detection of microorganisms in the field of diagnostic microbiology. Diagnostic oligonucleotide primers may target a wide variety of nucleic acid sequences, and attention has recently focused on the internal transcribed spacer (ITS) of the genes coding for 16S-23S rRNA (16S-23S rDNA), both for bacterial identification and detection applications (3, 8, 10) and for discrimination of clinical isolates within a species (11, 14).

In prokaryotes, the rRNA operon (*rrn*) contains 16S, 23S, and 5S rDNAs, and multiple *rrn* loci are frequently observed (12). The rRNA genes commonly occur in the order 16S-23S-5S, although there are frequent exceptions, and variations in the extent of repetition, arrangement, and linkage do occur among *rrn* loci. ITS length polymorphisms occur if the spacing that separates these genes varies and also if the ITS loci differ with respect to the number and type of tRNA genes contained within this region. Examples of this variability include *Bacillus subtilis*, with 10 *rrn* loci (12), and slowly growing species of *Mycobacterium* (5) and some *Mycoplasma* spp. (12) with only a single *rrn* locus. Of the pseudomonads, *Pseudomonas aeruginosa* has four rRNA operons organized in pairs of inverted repeats (19).

Although some investigators have attempted to use 16S rDNA sequence differences to differentiate bacteria at the genus and species levels (16), the 16S rDNA sequences have been found to be quite invariant when closely related organisms are compared and therefore they are not generally considered optimal targets for the development of differentiative species-specific probes. Barry et al. (3), however, demonstrated significant sequence heterogeneity in the 16S-23S rDNA ITSs in three *Clostridium* spp. and suggested that species-specific probes could be developed by targeting variable sequences in this region. In addition, 16S-23S rDNA ITS sequences were recently reported to be much more variable than those of 16S rDNA in a sequence-based differentiation of *Mycobacterium avium* complex, and the investigators were able to identify potential probe-primer sites for *M. avium* and *Mycobacterium intracellulare* (8).

PCR-based analysis of length polymorphisms in the 16S-23S rDNA ITS has also been previously suggested as a strategy for typing clinical isolates of both *Pseudomonas cepacia* (11) and *Neisseria meningitidis* (14). Furthermore, intergenic length polymorphisms in tRNA spacer regions are reported to have the potential to classify five species of *Staphylococcus* (24) and also to distinguish streptococcal strains and species (13).

In the present study, the rDNA ITSs of eight species of pseudomonads associated with human infections were cloned and sequenced in order to compare them and to identify variable sequences in this intergenic region with the prospect of developing species-specific primers for differentiation purposes.

MATERIALS AND METHODS

Bacterial strains and chemicals. The reference strains selected for this study were obtained from the American Type Culture Collection (ATCC) and are listed in Table 1. Clinical isolates of *P. aeruginosa* (101 specimens from 24 patients), *P. cepacia* (103 isolates from 65 patients), and *Pseudomonas pickettii*

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TABLE 1. ATCC reference strains used in evaluating the species specificities of primers targeting the ITS of 16S-23S rDNA

Organism	ATCC no.	Organism	ATCC no.
Comamonas testosteroni	11996	*Pseudomonas gladioli* subsp. *allicola*	19302
Pseudomonas aeruginosa	17933	*Pseudomonas mallei*	23344
	19660		
	27853	*Pseudomonas mendocina*	25411
Pseudomonas alcaligenes	14909	*Pseudomonas pickettii*	27511
Pseudomonas allicola	19302		27512
Pseudomonas caryophylli	25418	*Pseudomonas pseudoalcaligenes*	17440
Pseudomonas cepacia	17759	*Pseudomonas pseudomallei*	23343
	25415		
	25609	*Pseudomonas putida*	12633
Pseudomonas diminuta	11568	*Pseudomonas solanacearum*	11696
Pseudomonas fluorescens	13525	*Pseudomonas stutzeri*	17588
Pseudomonas gladioli subsp. *gladioli*	10248	*Pseudomonas vesicularis*	11426

(16 isolates from 16 patients) were collected between 1988 and 1993. With the exceptions of *Pseudomonas mallei* and *Pseudomonas pseudomallei*, all species were maintained by the National Laboratory for Bacteriology. Bacterial strains were grown overnight on Columbia blood agar base (Quelabs, Montreal, Quebec, Canada) at 37°C in 5% CO_2. Competent *Escherichia coli* DH5α was purchased from Gibco/BRL (Burlington, Ontario, Canada). Custom oligonucleotide primers, listed in Table 2, were either purchased from GSD Oligos (Toronto, Ontario, Canada) or synthesized with an ABI 392 DNA/RNA Synthesizer. Restriction endonucleases were purchased from Stratagene (La Jolla, Calif.) and *Taq* polymerase was purchased from Promega (Madison, Wis.), and they were used according to the manufacturers' protocols.

Nucleic acid isolation and PCR amplification of the 16S-23S rDNA ITS. Total nucleic acids were isolated from all bacterial strains by sodium dodecyl sulfate lysis followed by phenol-chloroform extraction and ethanol precipitation (20). Strains of *P. mallei* and *P. pseudomallei* were extracted directly from the ATCC lyophilized vials and were not cultured. Aliquots of 100 ng of total nucleic acids from each species were amplified in a 50-μl reaction mixture of the following composition: 200 μM each deoxynucleoside triphosphate, 1 μM primer A1 and A2 or B1 and B2, 2.5 U of *Taq* polymerase, and 1× reaction buffer supplied by the manufacturer, with an $MgCl_2$ concentration of 1.5 mM. The primer sequences used in this study are summarized in Table 2. PCR was performed in a PE 9600 (Perkin-Elmer Cetus) thermocycler using an initial denaturation of 2 min at 94°C followed by 30 cycles of 30 s at 94°C, 30 s at 68°C, and 1 min at 72°C. Following amplification, samples were incubated at 72°C for 10 min and then cooled to 4°C. PCR mixtures were analyzed by electrophoresis in 2% agarose and stained with ethidium bromide (20). Where indicated, the amplicons were digested with *Eco*RI and *Spe*I and subsequently purified by using PCR Magic Preps (Promega) according to procedures recommended by the manufacturer to desalt and remove excess primers. The amplicons were then cloned into the *Eco*RI-*Spe*I site of pBluescript SK^+ (Stratagene) and transformed into competent *E. coli* DH5α. The transformants were selected on Luria broth agar with ampicillin supplemented with IPTG (isopropyl-β-D-thiogalactopyranoside) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) to screen for the presence of inserts according to standard protocols (20). Between 10 and 20 individual clones were selected for sequence analysis. When multiple inserts of different sizes were observed, 8 to 12 clones of each size type were selected. The DNA sequences of the ITSs were determined either manually by using Sequenase version 2.0 (United States Biochemicals, Cleveland, Ohio) or with an ABI 373A automated sequencer and dye terminator chemistry. Analysis of the ITS sequences was carried out by using PC/GENE Release 6.6 (IntelliGenetics, Inc., Mountain View, Calif.), and alignment was performed by using the CLUSTAL program with final manual adjustment. Sequence polymorphisms were deemed to be real and not amplification artifacts only if they were identified in more than one clone. For the purpose of determining homology between the different ITSs, the sequences corresponding to the 16S and 23S rRNAs of *P. aeruginosa* were omitted during the comparison, and the sizes of the fragments given are those of the ITS region only. Percent homologies were determined by using the NALIGN program of PC/GENE. The ITSs within each species were designated as follows: Paer (*P. aeruginosa*), Pcep (*P. cepacia*), Pgla (*Pseudomonas gladioli*), Pmal (*P. mallei*), Pmen (*Pseudomonas mendocina*), Ppic (*P. pickettii*), Ppse (*P. pseudomallei*), and Xmal (*Xanthomonas maltophilia*). Each individual ITS sequevar was further designated numerically (e.g., Paer1 and Paer2).

Design and evaluation of species-specific probes. On the basis of the alignment of the various ITS sequences shown in Fig. 1, primers which targeted unique sequences within *P. aeruginosa*, *P. cepacia*, or *P. pickettii* were designed. As shown in Table 2, PA1 and PA2 target the *P. aeruginosa* ITS, PP1 and PP2 target the *P. pickettii* ITS, and PC1 and PC2 target the 23S rDNA of *P. cepacia*. The diagnostic potential and specificities of these primers were estimated by testing a total of 220 clinical isolates, including 101 strains of *P. aeruginosa*, 103 strains of *P. cepacia*, and 16 strains of *P. pickettii*, in addition to 24 ATCC reference strains of a variety of *Pseudomonas* species. PCR conditions were identical to those used to amplify the ITS region except that the annealing temperature was reduced to 60°C.

Nucleotide sequence accession numbers. The ITS sequences have been assigned GenBank accession no. L28148 (Paer1) through L28166 (Xmal1).

RESULTS

PCR amplification of the 16S-23S rDNA ITS. PCR primers A1 and A2 target conserved sequences flanking the 3′ end of the 16S and the 5′ end of the 23S rDNAs and were designed from an alignment of the 16S and 23S rDNA sequences from *P. aeruginosa* (21, 22) and *P. cepacia* (7, 9). rDNA ITSs were amplified in reference strains *P. aeruginosa* ATCC 19660, *P. cepacia* ATCC 25609, *P. gladioli* ATCC 10248, *P. mallei* ATCC 23344, *P. mendocina* ATCC 25411, *P. pickettii* ATCC 27511, *P. pseudomallei* ATCC 23343, and *X. maltophilia* ATCC 13637.

TABLE 2. Oligonucleotide primers used for amplification of the 16S-23S ITS region of pseudomonads and for identification to species level of *P. aeruginosa*, *P. cepacia*, and *P. pickettii*

Organism	Primer	Sequence	Target sequence	Amplicon size(s) (bp)
Pseudomonas sp.	A1	GCC CGT CAC ACC ATG GGA G	16S rDNA	Various (species dependent)
	A2	TCG CCT (G/C)TG (A/G)(A/G)G CCA AGG C	23S rDNA	
Pseudomonas sp.	B1	CGA CTA CTA GTG CCC GTC ACA CCA TGG GAG	16S rDNA	Various (species dependent)
	B2	CGA CTG AAT TCT CGC CT(G/C) TG(A/G) (A/G)GC CAA GGC	23S rDNA	
P. aeruginosa	PA1	TCC AAA CAA TCG TCG AAA GC	Paer1 and Paer2 ITSs	181
	PA2	CCG AAA ATT CGC GCT TGA AC		
P. cepacia	PC1	GCT GCG GAT GCG TGC TTT GC	23S rDNA	323
	PC2	GCC TTC TCC AAT GCA GCG AC		
P. pickettii	PP1	GGA AGA TGT TCT CTG CCG TGA	Ppic1 ITS	298
	PP2	TCG ATT GCT CTG GTA ATA CT		

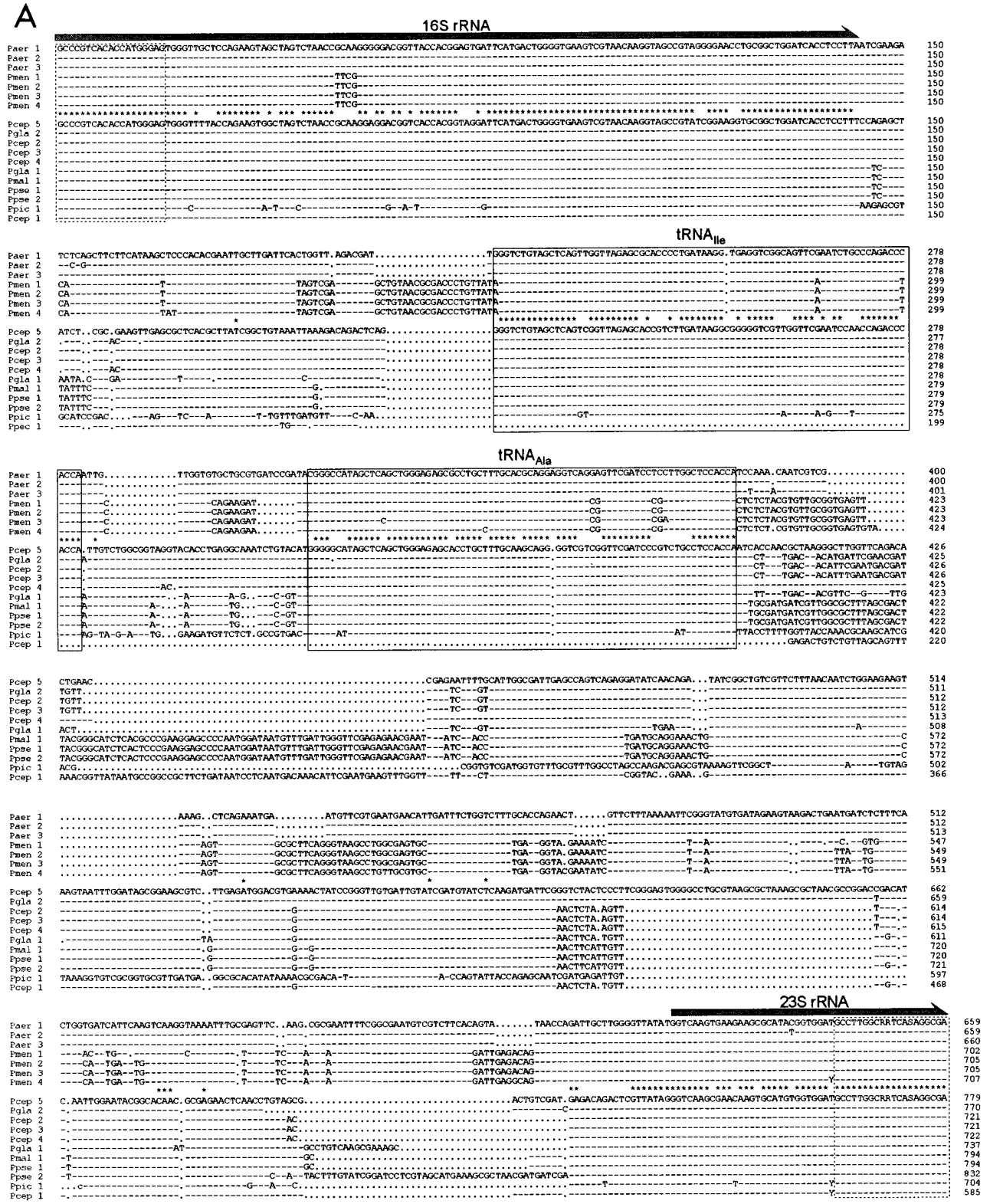

FIG. 1. Multiple nucleotide alignment of the 16S-23S rDNA ITS sequences of seven *Pseudomonas* spp. and nucleotide sequence of the 16S-23S rDNA ITS from *X. maltophilia*. The potential tRNA sequences for isoleucine and alanine (boxed), PCR primers (dashed boxes), regions corresponding to the 16S and 23S rDNAs of *P. aeruginosa* (arrows), identical bases within the homology group (dashes), gaps within the sequence (dots), and homologous bases in all spacers after multiple alignment (asterisks) are indicated. International Union of Pure and Applied Chemistry nomenclature was used to denote degenerate locations in the primers and 23S rDNA regions (R = A/G, S = G/C, and Y = T/C). (A) Sequence alignment for 16S-23S rDNA ITS regions of *P. aeruginosa* ATCC 19660 (Paer), *P. mendocina* ATCC 25411 (Pmen), *P. cepacia* ATCC 25609 (Pcep), *P. gladioli* ATCC 10248 (Pgla), *P. picketii* ATCC 27511 (Ppic), *P. pseudomallei* ATCC 23343 (Ppse), and *P. mallei* ATCC 23344 (Pmal). (B) The ITS sequence for *X. maltophilia* ATCC 13637. The location designated for the 16S rDNA sequence is based on an unpublished nucleotide sequence (GenBank accession no. M59158), and that of the 23S sequence was deduced from sequence homology with *P. aeruginosa.*

FIG. 1—*Continued.*

We observed little evidence of extensive ITS length polymorphisms in the representative strains of pseudomonads in this study, but sequence analysis of the amplified DNA revealed microheterogeneity within each ITS copy. The amplicons were therefore purified and cloned to effect their definitive sequencing. Empirical testing of a variety of restriction endonucleases was performed to identify those which would not cut within the ITS (data not shown). *Eco*RI and *Spe*I were selected, and primers B1 and B2 were designed accordingly to permit subsequent amplification and cloning into pBluescript.

Sequence analysis of the 16S-23S rDNA ITS. A multiple nucleotide sequence alignment of the 16S-23S rDNA ITSs of seven *Pseudomonas* spp. is presented in Fig. 1A, and the sequence of the *X. maltophilia* ITS is shown in Fig. 1B. The ITS homologies between species and among sequevars are summarized in Table 3 and clearly indicate that the spacers can be divided into two basic homology groups, one consisting of *P. aeruginosa* and *P. mendocina* and the other comprising *P. cepacia*, *P. gladioli*, *P. mallei*, *P. pickettii*, and *P. pseudomallei*, although *P. pickettii* is not as closely related to these species as they are to each other. The single, 536-bp 16S-23S ITS of *X. maltophilia* showed <50% sequence homology with any of the spacers from the other species and stands alone.

In the ATCC strains used in this study, five different 16S-23S rDNA ITS sequevars in *P. cepacia*, four in *P. mendocina*, three in *P. aeruginosa*, two each in *P. gladioli* and *P. pseudomallei*, and one each in *P. mallei*, *P. pickettii*, and *X. maltophilia* were identified. The range of ITS sizes observed among the ATCC strains was from 394 bp in *P. cepacia* to 641 bp in *P. pseudomallei. P. cepacia* had the largest variety of ITSs of any of the species studied, having five sequevars ranging in size from 394 to 588 bp, with three of the five ITSs essentially of identical lengths (530 and 531 bp).

Within species, the three ITS sequevars of *P. aeruginosa* and the two ITS sequevars of *P. pseudomallei* were found to have the highest degrees of homology, exceeding 98%, whereas more divergence was observed in the multiple ITS sequevars within the other species studied. For example, three of the four ITS sequevars of *P. mendocina* (Pmen2 [514 bp], Pmen3 [514 bp], and Pmen4 [516 bp]) demonstrated homologies ranging from 97.7 to 99.6%. Homologies of ITS Pmen1 (511 bp) were in the range of 94.3 to 96.3%. In the five *P. cepacia* ITS sequevars, Pcep2 (530 bp) and Pcep3 (530 bp) differed by only a single base, giving a relative homology of 99.8%, while ITS Pcep4 (531 bp) was most similar to Pcep5 (588 bp), with a homology of 95.7%, and slightly less similar to Pcep2 and Pcep3, with relative homologies of 93.8 and 94%, respectively. Some areas containing significant differences between the

TABLE 3. Lengths and nucleotide sequence homologies of 16S-23S rDNA ITSs for ATCC reference strains

Organism	Spacer	Size (bp)	% Homology																		
			Paer1	Paer2	Paer3	Pmen1	Pmen2	Pmen3	Pmen4	Pcep1	Pcep2	Pcep3	Pcep4	Pcep5	Pgla1	Pgla2	Pmal1	Ppse1	Ppse2	Ppic1	
P. aeruginosa	Paer1	468																			
	Paer2	468	99.6																		
	Paer3	469	99.8	99.4																	
P. mendocina	Pmen1	511	76.5	76.1	76.5																
	Pmen2	514	79.9	79.5	80.0	96.3															
	Pmen3	514	79.5	79.1	79.5	95.9	99.6														
	Pmen4	516	79.7	79.3	79.3	94.3	98.1	97.7													
P. cepacia	Pcep1	394	40.4	40.6	39.1	40.9	40.9	40.6	40.4												
	Pcep2	530	51.9	52.1	51.8	49.5	48.1	47.7	48.1	86.0											
	Pcep3	530	52.1	52.4	52.0	49.5	48.1	47.7	47.9	85.8	99.8										
	Pcep4	531	51.5	51.4	52.9	50.1	51.4	51.2	50.0	79.4	93.8	94.0									
	Pcep5	588	51.9	52.1	53.3	50.3	50.4	50.2	49.2	80.2	98.4	93.6	95.7								
P. gladioli	Pgla1	546	52.6	52.4	52.2	50.7	52.1	51.9	51.7	79.7	85.7	85.7	84.7	81.3							
	Pgla2	579	54.3	54.3	53.9	50.5	49.6	47.9	48.1	80.5	94.9	94.9	92.5	94.1	82.1						
P. mallei	Pmal1	603	52.4	52.1	52.9	52.1	54.1	54.1	53.9	81.5	86.8	86.6	86.6	78.6	85.5	78.1					
P. pseudomallei	Ppse1	603	52.4	52.1	52.9	52.1	54.1	54.1	53.9	81.5	86.8	86.6	86.6	78.6	85.5	78.1	99.8				
	Ppse2	641	56.8	56.6	57.4	54.2	53.3	53.1	52.5	79.4	87.0	87.0	86.8	76.2	86.3	77.0	98.3	98.5			
P. pickettii	Ppic1	514	53.0	51.7	51.8	48.3	47.1	46.9	46.3	51.5	65.4	65.2	63.8	64.6	62.1	62.5	65.4	65.4	65.4		
X. maltophilia	Xmal1	536	44.0	43.8	42.0	40.3	38.5	38.5	36.6	37.8	32.5	32.5	34.5	35.4	34.9	33.2	40.3	40.3	41.6	35.8	

Pcep4 and Pcep5 ITSs were highly conserved between the Pcep4, Pcep2, and Pcep3 spacers. ITS Pcep1 (394 bp) was the most divergent of this family, with overall homology to the other spacers ranging between 74.9 and 86.0%. The two ITSs in *P. gladioli*, Pgla2 (579 bp) and Pgla1 (546 bp), were relatively dissimilar, with only 82.1% relative homology, and, in fact, the Pgla2 sequevar showed a higher level of homology with the *P. cepacia* ITS (94.1 to 94.9% with Pcep2 through Pcep5) than with Pgla1. Pgla1 appeared equally divergent from the Pcep2 through Pcep5 sequevars, with homologies ranging from 81.3 to 85.9%.

The Pcep1, Pmen4, and Ppic1 ITSs were identified in two operons with a T/C polymorphism at a location corresponding to position 29 of the 23S rDNA of *P. aeruginosa*. On the basis of the frequencies with which the individual ITSs were recovered during the sequencing, it appears that Paer3 and Pcep2 are also present in two operons and that Pgla1 is probably present in three or four operons.

The ITSs from *P. mallei* and *P. pseudomallei* showed a very high level of homology with each other and may be considered a specific family. *P. mallei* was found to possess only one ITS, designated Pmal1, which was 603 bp long. *P. pseudomallei* had two ITSs, Ppse1 (603 bp) and Ppse2 (641 bp). ITS Pmal1 was virtually identical to the Ppse1 spacer, with only a single base differing between them and a relative homology of 99.8%. The Ppse2 ITS was also quite similar to these two spacers, with homologies to Pmal1 and Ppse1 of 98.3 and 98.5%, respectively.

tRNA sequences identified in the 16S-23S spacer regions. With the exception of the 394-bp ITS of *P. cepacia* (Pcep1), all of the *Pseudomonas* spacers contained potential tRNA sequences for both isoleucine and alanine. These regions were found to correspond well with the consensus structure of tRNAs (15), with two exceptions. The $tRNA^{Ile}$ sequences for *P. aeruginosa* and *P. mendocina* contain a single-base deletion which results in a 4-bp stem instead of a 5-bp stem for the anticodon loop. The $tRNA^{Ala}$ sequence in Pmen1 (514 bp) of *P. mendocina* contains an A-to-C base change at position 14 of the tRNA sequence. *X. maltophilia* was found to contain a potential tRNA sequence for alanine only.

Evaluation of species-specific primers. Initial testing of the primers targeting the ITS regions of *P. aeruginosa* and *P. pickettii* against the reference strains listed in Table 1 indicated that the primers were specific for the desired organisms. PA1-PA2 amplified only the three *P. aeruginosa* ATCC reference strains, and PP1-PP2 amplified only the two *P. pickettii* reference strains. However, while the *P. cepacia* ITS primers proved to be species specific, they detected only the ATCC 25609 strain used to obtain the sequence data and failed to amplify the other two reference strains. When these primers were tested against the 103 clinical samples, it was found that they detected only a certain percentage of isolates. Several primer pairs targeting different areas and different ITS sequences were subsequently designed. These alternate primer sets again proved to be specific for *P. cepacia*, but none was able to detect all of the *P. cepacia* isolates. The ITS regions from all of the *P. cepacia* isolates were amplified with the B1-B2 primer set and compared. It was found that there was some minor length polymorphisms within these samples; however, there did not appear to be any correlation between the ITS banding pattern and the ability to obtain positive amplification. The ITS region of *P. cepacia* was abandoned as a target for species identification, and primers which target the 23S rDNA were chosen. These primers were specific for *P. cepacia* and were also able to detect all of the isolates studied. When the *P. aeruginosa* and *P. pickettii* clinical samples were tested with the B1-B2 primer set, no length polymorphisms were seen. The 101 *P. aeruginosa* and 16 *P. pickettii* clinical samples studied were all positively identified with their respective primer sets.

DISCUSSION

ITS sequencing divides the organisms used in this study into several homology groups, one consisting of *P. aeruginosa* and *P. mendocina*, another comprising *P. cepacia*, *P. gladioli*, *P. mallei*, *P. pickettii*, and *P. pseudomallei*, and finally *X. maltophilia*. This division is consistent with the traditional rRNA homology grouping within the family *Pseudomonadaceae* (17). The ITS sequences from *P. mallei* and *P. pseudomallei* showed a very high level of homology, and they can therefore be considered particularly closely related. Conversely, the single, 536-bp 16S-23S ITS of *X. maltophilia* showed <50% homology with spacers from the other species, thereby confirming its more remote taxonomic and phylogenetic relationship.

P. aeruginosa is the type species of rRNA group I, which includes *P. mendocina* and other saprophytic and phytopathogenic fluorescent pseudomonads. With the exception of *P. aeruginosa* and, more recently, *P. mendocina* (2), most of the group I pseudomonads are found in soil and environmental sources and have not been associated with human disease. All of the other species in our study fall into rRNA homology group II, referred to as the pseudomallei group. Within this group, *P. cepacia*, *P. gladioli*, *P. mallei*, and *P. pseudomallei* fall into one DNA homology group and *P. pickettii* falls into another. ITS sequence data confirm this subdivision, with *P. pickettii* having less homology with the other species than they do among themselves. *X. maltophilia*, a representative of rRNA group V, also is differentiated from the others by ITS sequencing.

A number of the species included within rRNA group II have been associated with human infections, some as overt pathogens and others as opportunists. Although once considered nonpathogenic, *P. pickettii* is now a recognized opportunist (23) and has been linked to nosocomial infections (18). The same situation has been documented for *P. gladioli* (6) in cystic fibrosis patients.

On the basis of 16S rRNA sequences, DNA:DNA homology, cellular lipid and fatty acid composition, and phenotypic characteristics, a new genus, *Burkholderia*, has recently been proposed and validated (1, 25). Seven species of the genus *Pseudomonas* homology group II have been transferred to the new genus, with *Burkholderia cepacia* as the type species. Although ITS sequence homology data support this proposal, we have maintained the *Pseudomonas* basonym nomenclature to avoid confusion and maintain consistency between nucleotide ITS sequence accession numbers and ATCC culture designations until the new genus has gained wider acceptance.

An unusual observation in our study relates to the intra- and interspecies homologies of *P. gladioli* ITSs. Not only were Pgla1 and Pgla2 relatively dissimilar, with only 82.1% homology, but both ITS sequevars showed higher levels of homology to the four long ITSs of *P. cepacia* than to each other. The phylogenetic implications of these observations invite speculation, and more study may be warranted.

It is proposed that the ITS is under less selective pressure than rDNA sequences with a functional product and therefore that there may exist more variability in size and/or sequence, which could provide a basis for strain differentiation (10, 11, 14). Jensen et al. (10) have investigated a broad range of bacteria and suggest that ITS size polymorphisms may form a basis for PCR-based identification methods for many bacterial species. In the process of developing this suggestion for the

identification of *P. aeruginosa*, *P. cepacia*, and *P. pickettii*, it was found that *P. cepacia* possessed only minor size polymorphisms but appeared to have considerable sequence polymorphism within the ITS. We observed three different ITS amplification patterns, with the majority of the isolates having the same pattern as *P. cepacia* ATCC 25609, although no single primer pair targeting the *P. cepacia* ITS was successful in amplifying the ITSs of all of the clinical isolates studied. These results confirm those of Kostman et al. (11), who demonstrated size polymorphisms in the 16S-23S ITSs of 12 strains of *P. cepacia*, but we suggest that this is not as extensive as is required to be diagnostically useful. Primers developed in this study which target ITS sequences of *P. aeruginosa* and *P. pickettii* and 23S rDNA of *P. cepacia* have considerable potential for the early and rapid differentiation of isolates from respiratory and nosocomial infections. The ability of these diagnostic primers to detect small numbers of *P. cepacia* and *P. pickettii* organisms in sputum samples from patients with cystic fibrosis is being evaluated. The introduction of these genetically based methods for identification should help avoid the common misidentifications of *P. cepacia* as *Alcaligenes xylosoxidans* or *X. maltophilia*. Accurate isolate identification is particularly important in the management of cystic fibrosis patients who are colonized with *P. cepacia* in order to minimize transmission in social and clinical settings and to allow remedial chemotherapy.

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