

## Identification of *Pandoraea* Species by 16S Ribosomal DNA-Based PCR Assays

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The recently described genus *Pandoraea* contains five named species (*Pandoraea apista*, *Pandoraea pulmonicola*, *Pandoraea pnomenusa*, *Pandoraea sputorum*, and *Pandoraea norimbergensis*) and four unnamed genomospecies. *Pandoraea* spp. have mainly been recovered from the respiratory tracts of cystic fibrosis (CF) patients. Accurate genus- and species-level identification by routine clinical microbiology methods is difficult, and differentiation from *Burkholderia cepacia* complex organisms may be especially problematic. This can have important consequences for the management of CF patients. On the basis of 16S ribosomal DNA sequences, PCR assays for the identification of *Pandoraea* spp. were developed. A first PCR assay was developed for the identification of *Pandoraea* isolates to the genus level. PCR assays for the identification of *P. apista* and *P. pulmonicola* as a group, *P. pnomenusa*, *P. sputorum*, and *P. norimbergensis* were also developed. All five assays were evaluated with a panel of 123 bacterial isolates that included 69 *Pandoraea* sp. strains, 24 *B. cepacia* complex strains, 6 *Burkholderia gladioli* strains, 9 *Ralstonia* sp. strains, 5 *Alcaligenes xylosoxidans* strains, 5 *Stenotrophomonas maltophilia* strains, and 5 *Pseudomonas aeruginosa* strains. The use of these PCR assays facilitates the identification of *Pandoraea* spp. and avoids the misidentification of a *Pandoraea* sp. as a *B. cepacia* complex isolate.

Chronic microbial colonization of the large airways, leading to exacerbations of pulmonary infection, is the major cause of morbidity and mortality in patients with cystic fibrosis (CF). Typical pathogens of CF patients include *Burkholderia cepacia* complex organisms, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Haemophilus influenzae* (4, 7, 8). Other glucose nonfermenters such as *Stenotrophomonas maltophilia*, *Alcaligenes xylosoxidans*, *Ralstonia pickettii*, and *Burkholderia gladioli* can frequently be found as well (1, 4).

A recent polyphasic taxonomic study (2) showed that a number of bacterial isolates cultured from respiratory secretions of CF patients belonged to the new genus *Pandoraea*. Originally, this genus contained five named species (*Pandoraea apista* [the type species], *Pandoraea norimbergensis*, *Pandoraea pnomenusa*, *Pandoraea pulmonicola*, and *Pandoraea sputorum*) and one unnamed genomic species (2). More recently, three new (yet unnamed) *Pandoraea* genomospecies, previously classified as CDC weak oxidizer group 2, were added (3). *Pandoraea* spp. have mainly been isolated from the respiratory secretions of CF patients but have also been found in other clinical samples (including blood), soil, water, and food (2, 3, 11).

In the clinical microbiology laboratory, identification to the species level and differentiation of *Pandoraea* species from organisms belonging to the *B. cepacia* complex, *R. pickettii*, or *Ralstonia paucula* may be problematic (2, 5, 11). To aid in the identification of these organisms, we developed PCR-based identification strategies based on the 16S rRNA gene (rDNA). A first PCR assay was designed to identify all *Pandoraea* spp.

Subsequent PCR assays were designed to identify individual *Pandoraea* species.

### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** All isolates were obtained from the *Burkholderia cepacia* Research Laboratory and Repository (University of Michigan, Ann Arbor), the BCCM/LMG Bacteria Collection (Laboratorium voor Microbiologie, Universiteit Gent, Ghent, Belgium), or the Centers for Disease Control and Prevention (Atlanta, Ga.). Strains were grown aerobically on Mueller-Hinton broth (Becton Dickinson, Cockeysville, Md.) supplemented with 1.8% (wt/vol) agar and were incubated overnight at 32°C. For evaluation of the PCR assays, 69 *Pandoraea* isolates were used. Isolates were identified by a polyphasic taxonomic approach or DNA-DNA hybridizations, as described previously (2, 3). If serial isolates from a patient were available, only one isolate was included in the present study. We included 29 *P. apista* isolates (LMG 16407<sup>T</sup>, LMG 18818, LMG 18089, AU003, AU01287, AU0170, AU0871, AU1330, AU1430, AU1434, AU1465, AU1596, AU1656, AU1728, AU1776, AU2028, AU2160, AU2172, AU2303, AU2347, AU2377, AU2405, AU2513, AU2593, HI2743, HI2744, HI2782, CDC-G3307, and CDC-G9278), 5 *P. norimbergensis* isolates (LMG 18379<sup>T</sup>, LMG 13019, LMG 16603, R-4026, and AU1290), 3 *P. pulmonicola* isolates (LMG 18106<sup>T</sup>, LMG 18107, and LMG 18108), 15 *P. pnomenusa* isolates (LMG 18087<sup>T</sup>, LMG 18817, LMG 18820, AU1039, AU2170, AU2268, HI2279, HI2344, HI2778, HI2779, HI2780, HI2781, CDC-G5056, CDC-G7835, and CDC-G8107), 13 *P. sputorum* isolates (LMG 18819<sup>T</sup>, LMG 18100, AU0012, AU0103, AU0125, AU1359, AU1570, AU2075, AU2224, AU2269, AU2302, AU2389, and HI2742), and 1 isolate of each unnamed genomic species (*Pandoraea* genomospecies 1 R-5199, *Pandoraea* genomospecies 2 CDC-G5084, *Pandoraea* genomospecies 3 CDC-G9805, and *Pandoraea* genomospecies 4 CDC-H652).

We also included 54 isolates representing phylogenetically related species and other species that may be encountered in the sputum of CF patients: *B. cepacia* genomovar I (three isolates), *Burkholderia multivorans* (two isolates), *B. cepacia* genomovar III (seven isolates), *Burkholderia stabilis* (two isolates), *Burkholderia vietnamiensis* (two isolates), *B. cepacia* genomovar VI (five isolates), *Burkholderia ambifaria* (three isolates), *B. gladioli* (six isolates), *R. pickettii* (five isolates), *R. paucula* (two isolates), *Ralstonia gilardii* (two isolates), *P. aeruginosa* (five isolates), *A. xylosoxidans* (five isolates), and *S. maltophilia* (five isolates).

**DNA preparation.** DNA was prepared by heating one or two colonies (picked from a culture grown overnight) at 95°C for 15 min in 20 µl of lysis buffer containing 0.25% (vol/vol) sodium dodecyl sulfate and 0.05 M NaOH. Following

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TABLE 1. 16S rDNA-derived oligonucleotide primers used for PCR

Primer	Sequence (5'-3')	Target	Annealing temp (°C)	Nucleotide position	Product size (bp)
panF	GGGCTYAACTGGGAAGTGCATTC	Genus <i>Pandoraea</i>	68	584–607 <sup>a</sup>	645 <sup>a</sup>
panR	CGRYTTGGCRRCCCTCTGTACCG	Genus <i>Pandoraea</i>		1207–1229 <sup>a</sup>	
appuF <sup>b</sup>	CAGTGGGGGAATTTTGGACAATGGGCGCA	<i>P. apista</i> , <i>P. pulmonicola</i>	71	324–351 <sup>a</sup>	905 <sup>a</sup>
spuF	CTCGCGCTACAAGAGCGGCCGATA	<i>P. sputorum</i>	63	184–207 <sup>c</sup>	813 <sup>c</sup>
spuR	CGAGCACATCCGCCTCTCAGCAGAC	<i>P. sputorum</i>		973–997 <sup>c</sup>	
pnoF	CAGTGGGGGAATTTTGGACAATGGGCGCA	<i>P. pnomenusa</i>	71	313–339 <sup>d</sup>	673 <sup>d</sup>
pnoR	CGAGCACTCCACCTCTCAGCAGGA	<i>P. pnomenusa</i>		962–986 <sup>d</sup>	
norF	CAAGAGCAGCCGATGTCAGATTAGCTT	<i>P. norimbergensis</i>	66	193–219 <sup>e</sup>	804 <sup>e</sup>
norR	CGAGCACTCTCACCTTTCAGCAA	<i>P. norimbergensis</i>		975–997 <sup>e</sup>	

<sup>a</sup> Position and size relative to 16S rDNA sequence of *P. apista* LMG 18089 (AF139172).

<sup>b</sup> Used in combination with oligonucleotide primer panR.

<sup>c</sup> Position and size relative to 16S rDNA sequence of *P. sputorum* LMG 18819<sup>T</sup> (AF139176).

<sup>d</sup> Position and size relative to 16S rDNA sequence of *P. pnomenusa* LMG 18087<sup>T</sup> (AF139174).

<sup>e</sup> Position and size relative to 16S rDNA sequence of *P. norimbergensis* LMG 13019 (AF139171).

lysis, 180 µl of sterile distilled water was added to the lysis buffer and the DNA solutions were stored at –20°C.

**Development of primers for genus- and species-specific PCR assays.** Available 16S rDNA sequences of all *Pandoraea*, *Burkholderia*, and *Ralstonia* species were retrieved from the GenBank database and were aligned by using the MegAlign software package (DNASTar Inc., Madison, Wis.). On the basis of this alignment, the primers shown in Table 1 were developed.

**PCR.** PCR assays were performed in 25-µl reaction mixtures, containing 2 µl of DNA solution, 1 U of *Taq* polymerase (Gibco BRL, Gaithersburg, Md.), 250 mM (each) deoxynucleoside triphosphate (Gibco BRL), 5 µl of 5 M betaine (Sigma, St. Louis, Mo.), 1× PCR buffer (Qiagen, Valencia, Calif.), and 20 pmol of each oligonucleotide primer. Amplification was carried out with a PTC-100 programmable thermal cycler (MJ Research, Incline Village, Nev.). After initial denaturation for 2 min at 94°C, 20 amplification cycles were completed, each consisting of 1 min at 94°C, 45 s at the appropriate annealing temperature (Table 1), and 1 min at 72°C. A final extension of 10 min at 72°C was applied. Negative control PCRs with all components of the reaction mixture except template DNA were used for every experiment.

## RESULTS AND DISCUSSION

**Primer design.** Multiple-sequence alignment of the 16S rRNA genes of the different *Pandoraea* species revealed high similarity values. Several genus-level sequence signatures were detected, and these were incorporated into genus-specific primers panF (forward primer) and panR (reverse primer). Species-level sequence signatures were also detected, and specific PCR primers were developed for the identification of *P. apista* and *P. pulmonicola* (as a group) (forward primer appuF used in combination with reverse primer panR), *P. pnomenusa* (forward primer pnoF used in combination with reverse primer pnoR), *P. sputorum* (forward primer spuF used in combination with reverse primer spuR), and *P. norimbergensis* (forward primer norF used in combination with reverse primer norR) (Table 1). Figure 1 illustrates the results of the PCRs with these various primer pairs.

**Sensitivities and specificities of PCR assays.** Each of the 123 strains included in the present study was examined by PCR with the primer pairs mentioned above. The results are detailed in Table 2.

**Use of PCR assays for the identification of *Pandoraea* spp.** *B. cepacia* complex organisms are capable of chronic colonization of the respiratory tracts of CF patients and are associated with

increased rates of morbidity and mortality. To prevent the interpatient spread of *B. cepacia* complex organisms, strict infection control measures which have a significant impact on the quality of life of CF patients are recommended (7, 8). Accurate identification of *B. cepacia* complex bacteria is essential for infection control but is far from straightforward, and taxa that are frequently misidentified as belonging to the *B. cepacia* complex include *B. gladioli*, *R. pickettii*, *S. maltophilia*, and *Pandoraea* spp. (5, 6, 10, 12). Species belonging to the genus *Pandoraea* have mainly been isolated from CF patients worldwide, but other sources of isolation include blood, powdered milk, water, and sludge (2, 3, 11). Correct identification of these organisms is difficult and requires extensive biochemical testing or a polyphasic taxonomic approach, or both (2, 3, 5). Recently, several rRNA gene-based PCR assays have been developed for the identification of *B. cepacia* complex organisms (9, 15) and other pathogens of CF patients, including *S. maltophilia* (14) and *B. gladioli* (13).

To design PCR tests that would allow the identification of *Pandoraea* species, we sought genus-level and species-level signature sequences in the 16S rRNA gene. When available *Pandoraea* sp. 16S rRNA gene sequences were compared with sequences from representatives of the phylogenetically closely related genera *Burkholderia* and *Ralstonia*, several regions within the 16S rRNA gene were identified that showed enough diversity to allow the design of a primer pair (panF-panR) specific for the genus *Pandoraea*. As reported previously, comparison of the 16S rRNA gene sequences from all *Pandoraea* species revealed a high degree of identity (2, 3), resulting in few opportunities to design species-specific primers. The norF-norR primer pair allowed the specific amplification of a 16S rDNA fragment for all *P. norimbergensis* isolates investigated. Primer pair spuF-spuR allowed the amplification of a 16S rDNA fragment of all *P. sputorum* isolates investigated; this primer pair also reacted with *Pandoraea* genomospecies 2 and 3. By focusing on sequences shared by *P. apista* and *P. pulmonicola*, forward primer appuF was designed. When used in combination with genus-specific reverse primer panR, this primer was specific for *P. apista* and *P. pulmonicola* isolates

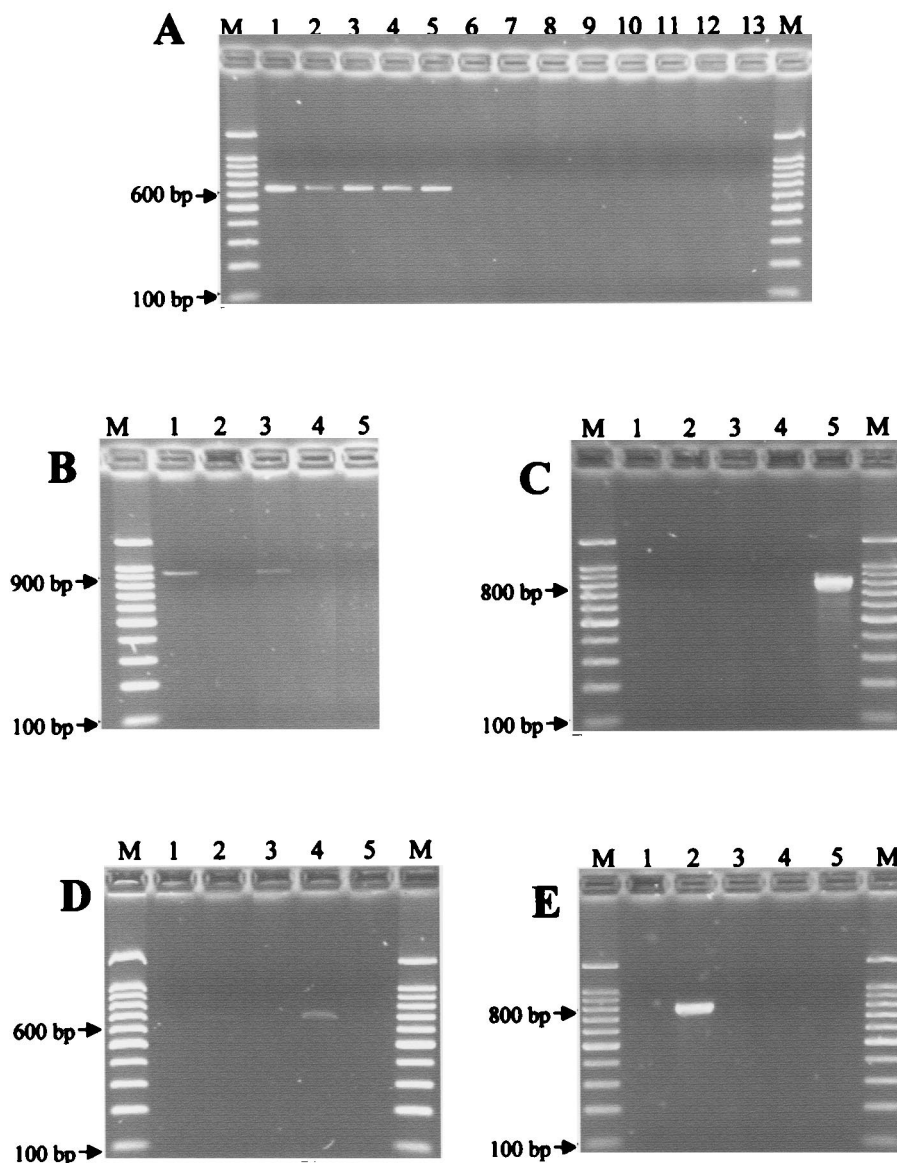


FIG. 1. PCR analysis of *Pandoraea* sp. type strains and other isolates with primer pairs panF-panR (A), appuF-panR (B), spuF-spuR (C), pnoF-pnoR (D), and norF-norR (E). Lanes: M, 100-bp DNA ladder (Gibco BRL); 1, *P. apista* LMG 16407<sup>T</sup>; 2, *P. norimbergensis* LMG 18379<sup>T</sup>; 3, *P. pulmonicola* LMG 18106<sup>T</sup>; 4, *P. pnomenusa* LMG 18087<sup>T</sup>; 5, *P. sputorum* LMG 18819<sup>T</sup>; 6, *B. cepacia* genomovar I LMG 1222<sup>T</sup>; 7, *B. cepacia* genomovar III HI2711; 8, *B. gladioli* AU1750; 9, *R. gilardii* LMG 5886<sup>T</sup>; 10, *R. paucula* LMG 3244<sup>T</sup>; 11, *P. aeruginosa* AU0225; 12, *S. maltophilia* AU0026; 13, *A. xylosoxidans* AU2116.

(this primer pair also reacted with one *B. cepacia* genomovar VI strain investigated). Primer pair pnoF-pnoR was designed for the specific amplification of a 16S rDNA fragment of *P. pnomenusa* isolates. Although this PCR assay shows excellent sensitivity, it also amplified a fragment of the 16S rDNAs of two *P. norimbergensis* isolates, one *P. apista* isolate, and *Pandoraea* genomospecies 4. Several isolates belonging to the *B. cepacia* complex reacted with this primer pair as well. However, since the genus-specific PCR assay and the assays for *P. apista* and *P. pulmonicola* as a group and for *P. norimbergensis* all displayed excellent specificities, the combined use of these assays will provide accurate identification of *P. pnomenusa* (the genus-specific primers will not react with *B. cepacia* complex

isolates, while the primers for *P. apista*-*P. pulmonicola* and *P. norimbergensis* will not react with *P. pnomenusa* isolates). Strain R-5199 (*Pandoraea* genomospecies 1) reacted only with the genus-specific primer pair panF-panR. We have not developed PCR assays specific for the unnamed *Pandoraea* genomic species, pending the availability of similar isolates.

Primer pair RHG-F (5'-GGGATTCATTCCTTAGTAA C-3') and RHG-R (5'-GCGACTACTAGCGATTCCAGC-3'), described by LiPuma et al. (9), was previously shown to be specific for *Burkholderia* and *Ralstonia* species. Since the genus *Pandoraea* occupies a phylogenetic position intermediate between the genera *Burkholderia* and *Ralstonia* (2), it is not surprising that all *Pandoraea* isolates investigated reacted pos-



TABLE 2. Results of PCR assays with different primer pairs

Primer pair and species tested	Sensitivity (%) <sup>a</sup>	Specificity (%) <sup>a</sup>	No. of strains for which result was <sup>b</sup> :	
			Positive	Negative
panF-panR				
<i>Pandoraea</i> sp.	99	100	68/69	1/69
All other species			0/54	54/54
spuF-spuR				
<i>P. sputorum</i>	100	98	13/13	0/13
All other species			2/110	108/110
norF-norR				
<i>P. norimbergensis</i>	100	100	5/5	0/5
All other species			0/118	118/118
appuF-panR				
<i>P. apista</i> and <i>P. pulmonicola</i>	97	99	31/32	1/32
All other species			1/91	90/91
pnoF-pnoR				
<i>P. pnomenusa</i>	100	82	15/15	0/15
Other <i>Pandoraea</i> species			4/54	50/54
All other species			16 <sup>c</sup> /54	38/54

<sup>a</sup> Overall values for each primer pair are given. The specificity of the assay with primer pair pnoF-pnoR for the genus *Pandoraea* was 93%.

<sup>b</sup> Values are numbers of strains for which result was obtained/numbers of strains tested.

<sup>c</sup> All 16 isolates were *B. cepacia* complex isolates.

itively with this primer pair (J. J. LiPuma, unpublished data). Isolates from the sputa of CF patients that are PCR positive with primer pair RHG-F–RHG-R but negative in PCR assays for *B. cepacia* complex organisms (9, 15) could belong to the genus *Pandoraea* and can be further investigated with primers specific for the genus *Pandoraea* described in this report.

In summary, the combined use of the PCR assays described here allows the separation of members of the genus *Pandoraea* from closely related genera. It also allows the accurate identification of all five named *Pandoraea* species. The use of these assays can significantly reduce the misidentification of *Pandoraea* spp. as *B. cepacia* complex isolates and by so doing will be an important adjunct in the evaluation of isolates found in cultures of sputa from CF patients.

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