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# Use of Amplified Ribosomal DNA Restriction Analysis for Identification of *Ralstonia* and *Pandoraea* Species: Interest in Determination of the Respiratory Bacterial Flora in Patients with Cystic Fibrosis

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**The recovery of *Ralstonia* and *Pandoraea* species from respiratory tract cultures of patients with cystic fibrosis has recently been reported. These species are difficult to identify, and especially to differentiate from *Burkholderia cepacia* complex organisms, with classical methods. The discriminatory power of amplified ribosomal DNA restriction analysis (ARDRA) within the two genera was assessed by comparing the restriction profiles of reference strains of each species by using a panel of six enzymes already proven suitable for the identification of *Burkholderia* species. ARDRA provided differentiation of all the *Ralstonia* species tested and of *Pandoraea norimbergensis*. *Pandoraea* species *P. pnomenusa*, *P. sputorum*, *P. pulmonicola*, and *P. apista* were not discriminated to the species level. This method allowed the identification of five clinical isolates recovered from French cystic fibrosis patients as *Ralstonia mannitolilytica*.**

The recovery of various gram-negative bacilli, mainly *Ralstonia* and *Pandoraea* species, from the sputa of patients with cystic fibrosis (CF) on *Burkholderia cepacia*-selective media has been recently pointed out (4, 5). The genus *Ralstonia*, described in 1995 by Yabuuchi et al.(16), contains environmental gram-negative bacilli including major plant pathogen *R. solanacearum* (12); potential bioremediation agents *R. eutropha* and the metal-resistant *R. campinensis*, *R. metallidurans*, and *R. basilensis* (11); and opportunistic human pathogens. Among strains of clinical importance, four *Ralstonia* species have been isolated from CF patients: *R. pickettii* (formerly *Pseudomonas pickettii* and *Burkholderia pickettii*) (2), *R. mannitolilytica* (formerly *R. pickettii* biovar 3/*thomasii*) (5, 10), and quite rarely *R. gilardii* (8) and *R. taiwanensis* (3, 8), whereas *R. paucula* (formerly CDC group IV c-2) has not been involved in CF to date. The novel genus *Pandoraea* (4) contains five species: *P. pnomenusa*, *P. sputorum*, *P. pulmonicola*, and *P. apista*, mainly associated with clinical isolates recovered from CF patients, and *P. norimbergensis* (formerly *Burkholderia norimbergensis*), rarely isolated from the environment and from human clinical specimens. Four *Pandoraea* genomospecies, containing one strain each, were also described (9). The misidentification of *Ralstonia* and *Pandoraea* species as organisms belonging to the *B. cepacia* complex (Bcc) is fraught with consequences since the recovery of a Bcc organism in a CF patient leads to strict infection control measures in order to reduce patient-to-patient spread, and possibly to exclusion from lung transplantation. At present, the prevalence, patient-to-patient transmissibility, and clinical impact of *Ralstonia* and *Pandoraea* organisms in CF patients are unknown and cannot be evaluated if they are badly recognized in the clinical microbiology laboratory. Thus, an accurate identification of these species is essential. Unfortunately, the usual manual and automated phenotypic identification methods are not satisfactory (1, 14, 15). The aim of this study was to determine whether amplified ribosomal DNA restriction analysis (ARDRA), already successfully applied to the identification of *Burkholderia* species (13), could be used for *Ralstonia* and *Pandoraea* species.

The type strains of the following *Ralstonia* and *Pandoraea* species were tested: *R. solanacearum*, *R. eutropha*, *R. campinensis*, *R. metallidurans*, *R. basilensis*, *R. pickettii*, *R. mannitolilytica*, *R. gilardii*, *R. taiwanensis*, and *R. paucula* and *P. pnomenusa*, *P. sputorum*, *P. pulmonicola*, *P. apista*, and *P. norimbergensis*. As *R. solanacearum* is known to be a heterogeneous species, several strains, representative of races 1, 2, and 3, kindly provided by Christian Boucher (Centre National de la Recherche Scientifique-Institut National de la Recherche Agronomique, Castanet-Tolosan, France) were included. The other reference strains were obtained from international culture collections. Five clinical isolates recovered from the sputum of CF patients and transmitted to the French Observatoire Cepacia/Vaincre la Mucoviscidose were also analyzed. These isolates had been grown on *B. cepacia*-selective media and were resistant to colistin, aminoglycosides, ticarcillin, and aztreonam but did not belong to the *Burkholderia* genus according to ARDRA. Two of these isolates, kindly provided by A. Ferroni, had been identified as *R. mannitolilytica* by use of 16S rRNA sequencing (10). The bacterial strains studied and their sources are listed in Table 1.

The restriction fragment length polymorphism (RFLP) analysis of amplified 16S rDNA was performed as previously described (13). Briefly, the primers fD1 (5′-CCGAATTCG TCGACAACAGAGTTTGATCCTGGCTCAG-3′) and rD1 (5′-CCCGGGATCCAAGCTTAAGGAGGTGATCCAGC C-3′) were used to amplify approximately 1,500 bp within

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TABLE 1. List of bacterial strains studied and ARDRA results[c]

| Strain or isolate | Source of isolation | ARDRA pattern for: | | | | | | ARDRA group |
|---|---|---|---|---|---|---|---|---|
| | | *Alu*I | *Cfo*I | *Dde*I | *Msp*I | *Nci*I | *Bss*KI | |
| Reference strains | | | | | | | | |
| *R. solanacearum* | | | | | | | | |
| Race 1 K 60$^T$/UW 25[a] | Tomato | H | D | D | H | J | A | R1 |
| Race 1 GMI 1000[a] | Tomato | K | D | D | H | J | A | R2 |
| Race 2 S 210/UW 70[a] | Plantain | H | D | D | H | J | A | R1 |
| Race 3 S 206/UW 80[a] | Potato | I | D | D | H | J | A | R3 |
| Race 3 S 207/UW 81[a] | Potato | I | D | D | H | J | A | R3 |
| *R. eutropha* LMG 1199$^T$ | Soil | L | F | L | D | L | L | R4 |
| *R. campinensis* LMG 19282$^T$ | Zinc desert | H | F | D | I | K | I | R5 |
| *R. metallidurans* LMG 1195$^T$ | Wastewater zinc factory | H | H | D | I | J | A | R6 |
| *R. basilensis* LMG 18990$^T$ | Freshwater pond | L | F | L | D | D | N | R7 |
| *R. pickettii* ATCC 27511$^T$ | Human (tracheotomy) | D | D | D | D | D | D | R8 |
| *R. mannitolilytica* LMG 6866$^T$ | Outbreak of hospital infection | K | D | D | H | K | A | R9 |
| *R. gilardii* LMG 5886$^T$ | Whirlpool | H | G | D | I | J | I[b] | R10 |
| *R. taiwanensis* LMG 19424$^T$ | Mimosa | H | K | L | I | K | I[b] | R11 |
| *R. paucula* LMG 3244$^T$ | Human (respiratory tract) | H | K | D | I | J | A | R12 |
| *P. pnomenusa* LMG 18087$^T$ | Human (CF patient, United Kingdom) | H | L | K | B | J | M | P1 |
| *P. sputorum* LMG 18819$^T$ | Human (CF patient, United States) | H | L | K | B | J | M | P1 |
| *P. pulmonicola* LMG 18106$^T$ | Human (CF patient, Canada) | H | J | K | B | J | M | P2 |
| *P. apista* LMG 16407$^T$ | Human (CF patient, Denmark) | H | J | K | B | J | M | P2 |
| *P. norimbergensis* LMG 18379$^T$ | Water layer, Germany | J | I | M | B | J | M | P3 |
| *B. cepacia* genomovar III LMG 12615 | Human (CF patient, United Kingdom) | A | A | A | A | A | A | |
| *B. multivorans* LMG 13010$^T$ | Human (CF patient, Belgium) | A | A | I | A | A | AI | |
| Clinical isolates | | | | | | | | |
| 5-6 | Human (CF patient, France) | K | D | D | H | K | A | R9 |
| 5-51 | Human (CF patient, France) | K | D | D | H | K | A | R9 |
| 5-52 | Human (CF patient, France) | K | D | D | H | K | A | R9 |
| 6-81 | Human (CF patient, France) | K | D | D | H | K | A | R9 |
| 9-26 | Human (CF patient, France) | K | D | D | H | K | A | R9 |

[a] Strain provided by C. Boucher, INRA, Castanet-Tolosan, France.
[b] Subtle difference in the size of one fragment compared to the I pattern.
[c] Results for the Bcc species were previously published (13).

the 16S rRNA gene. The PCR products were digested with the following endonucleases: *Alu*I, *Cfo*I, *Dde*I, *Msp*I, *Nci*I, and *Bss*KI.

Some of the restriction profiles obtained are shown in Fig. 1, and ARDRA results are summarized in Table 1. The previously published results (13) obtained for the two main Bcc species involved in cystic fibrosis, i.e., *B. cepacia* genomovar III and *B. multivorans*, are included in Table 1 for comparison. The 14 *Ralstonia* type and reference strains tested, representing 10 species, were classified in 12 ARDRA groups; the 5 *R. solanacearum* strains were classified in 3 ARDRA groups, and one of the strains belonging to race 1 was not discriminated from the representative strain of race 2 tested. The type strains of all the *Ralstonia* species included in this study harbored specific ARDRA patterns. The five *Pandoraea* type strains tested were classified in three ARDRA groups; thus, *P. pnomenusa* and *P. sputorum* on one hand and *P. pulmonicola* and *P. apista* on the other hand harbor the same RFLP profiles. The five clinical strains tested presented the same RFLP profiles as the *R. mannitolilytica* type strain. Finally, the ARDRA patterns clearly differentiate *Ralstonia* and *Pandoraea* species from *Burkholderia* species, as well as from other nonfermenting gram-negative bacilli frequently recovered from patients with CF, such as *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, and *Achromobacter xylosoxidans* (data not shown). The prevalence of *Ralstonia* species in patients with CF is probably low but may be underestimated due to misidentification. Coenye et al. recently reported that 42 of about 4,000 bacterial isolates collected from American CF patients and sent to the *Burkholderia cepacia* Research Laboratory and Repository (University of Michigan) belonged to the genus *Ralstonia* (8), with *R. mannitolilytica* being the most prevalent species (25 out of 42 isolates). In a French pediatric CF center, *R. mannitolilytica* was recovered from 2 out of 259 patients (10). Finally, 5 clinical isolates out of 247 nonredundant isolates recovered from 243 French CF patients and collected by the Observatoire Cepacia/Vaincre la Mucoviscidose, including the two isolates mentioned above, were identified as *R. mannitolilytica* in the present study. The pathogenic potential of this species is not clear; however, chronic colonization is possible. *Pandoraea* species have been recovered from CF patients in Canada, Denmark, Brazil, the United States, and the United Kingdom, but to our knowledge there are no available epidemiological data concerning the prevalence of these organisms in CF patients.

Phenotypic tests for *Ralstonia* and *Pandoraea* species can be misleading: confusion of *Ralstonia* sp. with *Burkholderia* sp., *Pseudomonas* sp., and *S. maltophilia* (8, 10) and of *Pandoraea*

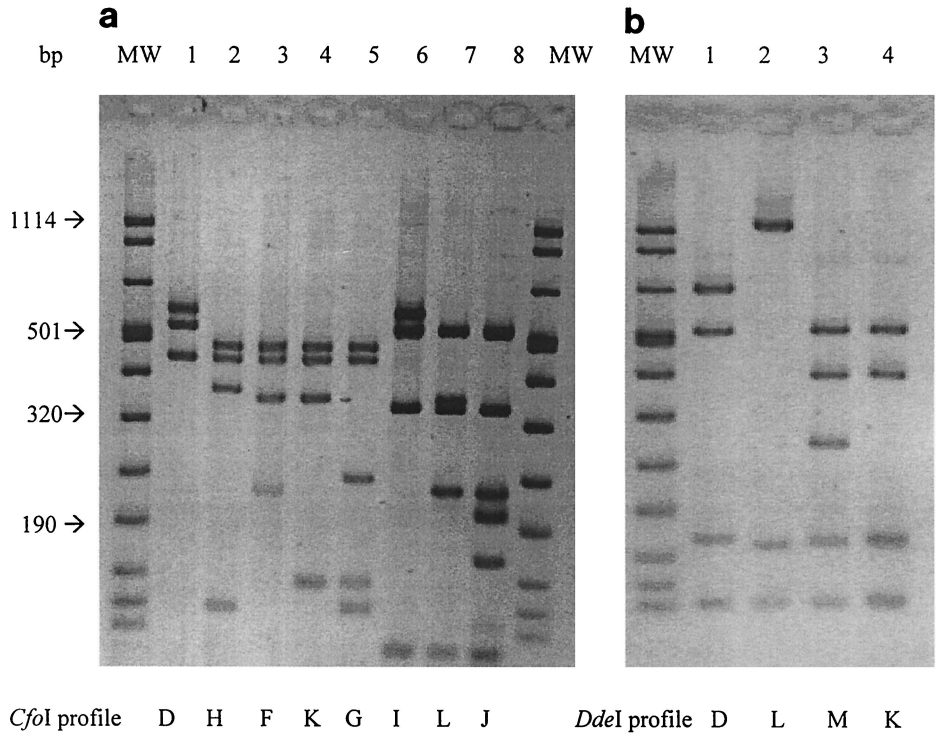


FIG. 1. ARDRA patterns. MW, molecular weight marker VIII (Roche). (a) Different *Cfo* I restriction profiles obtained within *Ralstonia* (lanes 1 to 5) and *Pandoraea* species (lanes 6 to 8). (b) Different *Dde* I restriction profiles obtained within *Ralstonia* (lanes 1 and 2) and *Pandoraea* species (lanes 3 and 4).

sp. with *Burkholderia* sp. and *Ralstonia* sp. has been reported (4). Thus, molecular methods are necessary for an accurate diagnosis. 16S rRNA gene sequencing (10) is widely applicable to any problematic isolate but requires a sequencer; 16S ribosomal DNA-based genus- and/or species-specific PCR has been developed for *R. mannitolilytica* and *R. pickettii* (8) and for *Pandoraea* species (7). Analysis of the *gyrB* gene seems promising for the identification of *Pandoraea* species (6). The ARDRA method tested in this study, though unable to separate all *Pandoraea* species, proves to be a useful identification tool; it requires the constitution of a data bank of profiles but has the advantage of being equally applicable to the main organisms growing on *B. cepacia*-selective media, i.e., *Burkholderia*, *Ralstonia*, and *Pandoraea*.

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