Description of *Pandoraea* gen. nov. with *Pandoraea apista* sp. nov., *Pandoraea pulmonicola* sp. nov., *Pandoraea pnomenusa* sp. nov., *Pandoraea sputorum* sp. nov. and *Pandoraea norimbergensis* comb. nov.

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A polyphasic taxonomic study was performed on a group of isolates tentatively identified as *Burkholderia cepacia*, *Ralstonia pickettii* or *Ralstonia paucula* (formerly known as CDC group IVc-2). The isolates were mainly cultured from sputum of cystic fibrosis patients or from soil. SDS-PAGE of whole-cell proteins and AFLP fingerprinting distinguished at least five different species, and this was confirmed by DNA–DNA hybridizations. 16S rDNA sequence analysis of representative strains indicated that these organisms belong to the β -subclass of the *Proteobacteria*, with the genera *Burkholderia* and *Ralstonia* as closest neighbours. Based on genotypic and phenotypic characteristics, the organisms were classified in a novel genus, *Pandoraea*. The DNA base composition of the members of the new genus is between 61.2 and 64.3 mol%. This novel genus includes four new species, *Pandoraea apista* (the type species) (type strain is LMG 16407^T), *Pandoraea pulmonicola* (type strain is LMG 18106^T), *Pandoraea pnomenusa* (type strain is LMG 18087^T) and *Pandoraea sputorum* (type strain is LMG 18819^T), and *Pandoraea norimbergensis* (Wittke et al. 1997) comb. nov. (type strain is LMG 18379^T). The available clinical data indicate that at least some of these organisms may cause chronic infection in, and can be transmitted amongst, cystic fibrosis patients.

Keywords: *Pandoraea*, *Burkholderia*, cystic fibrosis, *Ralstonia*, taxonomy

INTRODUCTION

The majority of life-threatening bacterial lung infections in cystic fibrosis (CF) patients are caused by a limited spectrum of bacterial pathogens which include *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Burkholderia cepacia* (Gilligan, 1991; Govan & Deretic, 1996). Other organisms which can be found regularly in respiratory tract specimens of CF patients include *Haemophilus influenzae*, *Stenotro-*

phomonas maltophilia, certain fungi (e.g. *Aspergillus* spp.) and viruses (e.g. respiratory syncytial virus) (Gilligan, 1991). In the absence of epidemic spread, *B. cepacia* only infects a small proportion of CF patients, but it has a major impact on both morbidity and mortality of infected patients and the medical and psychosocial implications of *B. cepacia* colonization and segregation of colonized and non-colonized patients as a means of infection control are enormous (LiPuma, 1998). Accurate identification of *B. cepacia* is crucial in patient management and infection control practices, but may be problematic, and misidentification is relatively common (LiPuma, 1998).

The present study was initiated by the receipt of several isolates cultured from sputa of CF patients in

Abbreviation: CF, cystic fibrosis.

The GenBank accession numbers for the 16S rRNA gene sequences determined in this work are: LMG 18819^T, AF139176; LMG 13019, AF139171; LMG 18089, AF139172; LMG 16407^T, AF139173; LMG 18087^T, AF139174; and LMG 18106^T, AF139175.

Denmark, Canada, the USA and Brazil. These strains were tentatively identified as *B. cepacia* or *Ralstonia pickettii*-like organisms or as *Ralstonia paucula* (formerly known as Centers for Disease Control group IVc-2; Vandamme *et al.*, 1999). This report describes the polyphasic taxonomic study which was used for the characterization of these and similar isolates from the environment and human clinical specimens. The genotypic and phenotypic characteristics warranted the classification of the isolates in a novel genus, for which we propose the name *Pandoraea*. The genus *Pandoraea* contains five species: *Pandoraea apista* sp. nov. (the type species), *Pandoraea pulmonicola* sp. nov., *Pandoraea pnomenusa* sp. nov., *Pandoraea sputorum* sp. nov. and *Pandoraea norimbergensis* (Wittke *et al.* 1997) comb. nov. (basonym *Burkholderia norimbergensis*). The name *Burkholderia norimbergensis* was proposed by Wittke *et al.* (1997) for a single bacterial isolate capable of heterotrophic sulfur oxidation, which was isolated from an oxic water layer above a sulfide-containing sediment.

METHODS

Bacterial strains and growth conditions. Strains used in this study are listed in Table 1. All strains were grown aerobically on Trypticase Soy Agar (BBL) at 37 °C unless otherwise indicated.

Phenotypic characterization. Classical phenotypic tests were performed as described previously (Vandamme *et al.*, 1993). API ZYM and API20 NE tests were performed according to the recommendations of the manufacturer (bioMérieux). Flagella staining was performed using a solution of tannic acid (2 g)/phenol (5%, w/v)/KAl(SO₄)₂ (saturated) in 11 ml saturated crystal violet in 95% ethanol. Stained cells were viewed by phase-contrast microscopy.

Phytopathogenicity was tested as described by Gonzalez & Vidaver (1979). In brief, strains were grown for 24 h at 28 °C. Bacterial suspensions containing 10⁹ c.f.u. ml⁻¹ were prepared in sterile distilled water and 100 µl of the suspension was inoculated on 3–5 mm thick onion slices. The inoculated slices were incubated for 48 h at 28 °C in moist chambers. *B. cepacia* genomovar I strain LMG 1222^T was used as a positive control. Sterile distilled water was used as a negative control.

Growth on *B. cepacia*-selective agar (DM253; Mast Diagnostics) containing ticarcillin (100 mg l⁻¹) and polymyxin B (300 000 U l⁻¹) was tested by incubating the strains at 37 °C for 48 h. *B. cepacia* genomovar III LMG 16656 was used as a positive control.

Degradation of poly-3-hydroxybutyrate (PHB) was tested by growing the strains on minimal Delafield medium containing 0.005% (w/v) yeast extract, 0.1% (w/v) NH₄Cl, 0.05% (w/v) MgSO₄·7H₂O, 0.005% (w/v) ferric ammonium citrate and 2.0% (w/v) agar in 0.033 M KH₂PO₄/Na₂HPO₄ buffer pH 6.8 and covering with a layer of PHB agar containing 2.5% (w/v) PHB and 2.0% agar. Strains were incubated at 37 °C for 7 d. *Acidovorax facilis* PHA 774 and *Pseudomonas balearica* PHA 785 were used as positive controls. Degradation of PHB was recognized by the appearance of clear haloes surrounding the colonies.

SDS-PAGE of whole-cell proteins. Strains were grown on nutrient agar containing 0.1% (w/v) beef extract, 0.2% (w/v) yeast extract, 0.5% (w/v) NaCl, 0.5% (w/v) peptone, 0.04% (w/v) KH₂PO₄, 0.24% (w/v) Na₂HPO₄·12H₂O and 2.0% agar (pH 6.8) for 48 h at 28 °C. Preparation of whole-cell proteins and SDS-PAGE were performed as described previously (Pot *et al.*, 1994). Densitometric analysis, normalization and interpolation of the protein profiles, and numerical analysis using the Pearson product moment correlation coefficient were performed using the GelCompar 4.2 software package (Applied Maths).

DNA preparation. This was done as described by Pitcher *et al.* (1989).

AFIP fingerprinting. The preparation of template DNA (using restriction enzymes *Apal* and *TaqI*), amplification [using primers A00 and T00 in the preselective PCR and primers B07 (labelled with the fluorescent dye 6-FAM) and T11 in the selective PCR], separation of the fragments using an ABI Prism 377 automated DNA sequencer and numerical analysis were performed as described previously (Coenye *et al.*, 1999b).

16S rDNA sequencing. The nearly complete (corresponding to positions 8–1541 in the *Escherichia coli* numbering system) 16S rRNA gene of strains LMG 18819^T, LMG 18087^T, LMG 13019, LMG 18106^T, LMG 18089 and LMG 16407^T was amplified by PCR using conserved primers [5'-AGAG-TTGATCCTGGCTCAG-3' (5'-CTGGCTCAGGAC/TGAACGCTG-3' for LMG 18087^T) and 5'-AAGGAGG-TGATCCAGCCGCA-3']. The PCR products were purified using a QIAquick PCR Purification kit (Qiagen) according to the manufacturer's instructions. Sequence analysis was performed using an Applied Biosystems 377 DNA Sequencer and the protocols of the manufacturer (Perkin-Elmer Applied Biosystems) using the ABI Prism Dye or BigDye Terminator Cycle Sequencing Ready Reaction kit. The sequencing primers were those described by Coenye *et al.* (1999a) except that primer *R was replaced by *R2 (5'-GCTACACACTGCTACAATG-3'). Sequence assembly was performed by using the program AutoAssembler 1.4.0 (Perkin-Elmer Applied Biosystems). Phylogenetic analysis was performed using the GeneCompar 2.1 software package (Applied Maths). A phylogenetic tree was constructed based on the neighbour-joining method (Saitou & Nei, 1987).

Determination of the DNA base composition. DNA was enzymically degraded into nucleosides as described by Mesbah *et al.* (1989). The obtained nucleoside mixture was then separated by HPLC using a Waters SymmetryShield C8 column thermostated at 37 °C. The solvent was 0.02 M NH₄H₂PO₄ pH 4.0 with 1.5% acetonitrile. Non-methylated lambda phage DNA (Sigma) was used as the calibration reference.

DNA-DNA hybridizations. These were performed with photobiotin-labelled probes in microplate wells as described by Ezaki *et al.* (1989), using an HTS7000 Bio Assay Reader (Perkin-Elmer) for the fluorescence measurements. The hybridization temperature was 50 °C. Reciprocal experiments were performed for every pair of strains and each value given is the mean of at least two hybridization experiments. The variation between reciprocal reactions and repeated experiments is around 7% with this method (Goris *et al.*, 1998).

Fatty acid methyl ester analysis. After an incubation period of 24 h at 35 °C, a loopful of well-grown cells was harvested

Table 1. List of strains studied

CCUG, Culture Collection, University of Göteborg, Department of Clinical Bacteriology, Göteborg, Sweden; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; LMG, BCCM/LMG Bacteria Collection Laboratorium voor Microbiologie Gent, Universiteit Gent, Belgium; NCTC, National Collection of Type Cultures, London, UK.

Species and strain designation	Other strain designations	Depositor	Source
<i>Burkholderia cepacia</i> genomovar I LMG 1222 ^T	NCTC 10743 ^T	B. Contopoulos	<i>Allium cepa</i>
<i>Burkholderia cepacia</i> genomovar III LMG 16656	J2315	Own isolate	CF patient (UK, 1989)
<i>Burkholderia cepacia</i> genomovar IV LMG 13017		S. Lauwers	Blood culture (Belgium, 1988)
<i>Burkholderia multivorans</i> LMG 13010 ^T	NCTC 13007 ^T	S. Lauwers	CF patient (Belgium, 1992)
<i>Burkholderia vietnamiensis</i> LMG 10929 ^T	TVV 75 ^T	T. Heulin	Rice rhizosphere (Vietnam)
<i>Pandoraea norimbergensis</i> LMG 18379 ^T	CCUG 39188 ^T , DSM 11628 ^T	DSM	Oxic water layer above a sulfide-containing sediment (Nürnberg, Germany)
LMG 13019	CCUG 38411	S. Lauwers	Blood culture (Belgium, 1990)
LMG 16603	CCUG 34867	Own isolate	Human broncheal alveolar lavage (Sweden)
R-4026	CCUG 39677	J. Moore	Powdered milk
<i>Pandoraea pulmonicola</i> LMG 18106 ^T LMG 18107 LMG 18108	CCUG 38759 ^T CCUG 38779 CCUG 38745	D. Speert J. LiPuma J. LiPuma	CF patient (Canada) CF patient (USA) CF patient (USA)
<i>Pandoraea pnomenusa</i> LMG 18087 ^T LMG 18820 R-1454 LMG 18817 R-4805 R-5620	CCUG 38742 ^T CCUG 39683 CCUG 39672 CCUG 39689 CCUG 39694 355	Own isolate J. Mergaert D. Speert J. LiPuma J. LiPuma S. Canani	CF patient (UK) Sludge (Germany) CF patient (Canada, 1996) CF patient (USA) CF patient (USA) CF patient (Brazil, 1997)
<i>Pandoraea apista</i> LMG 16407 ^T LMG 16408 LMG 16409 LMG 16410 LMG 16411 LMG 18089 R-2394 R-2395 R-2441 R-2885 R-3202 LMG 18818 R-4520 R-4527 R-4529 R-6145	CCUG 38412 ^T CCUG 38413 13037 13038 13720 CCUG 38750 CCUG 39675 CCUG 39674 CCUG 39679 CCUG 39681 CCUG 39676 CCUG 39687 CCUG 39688 CCUG 39690 CCUG 39686 AU0786	N. Høiby N. Høiby N. Høiby N. Høiby N. Høiby D. Speert J. LiPuma J. LiPuma J. LiPuma J. LiPuma J. LiPuma J. LiPuma J. LiPuma J. LiPuma J. LiPuma J. LiPuma	CF patient (Denmark) CF patient (Denmark) CF patient (Denmark) CF patient (Denmark) CF patient (Denmark) CF patient (USA) CF patient (USA) CF patient (USA) CF patient (USA) CF patient (USA) CF patient (USA) CF patient (USA) CF patient (USA) CF patient (USA) CF patient (USA) CF patient (USA)
<i>Pandoraea sputorum</i> LMG 18819 ^T LMG 18100 R-2456 R-2711 R-2886 R-2398 R-3080	CCUG 39682 ^T CCUG 38753 CCUG 39685 CCUG 39691 CCUG 39684 CCUG 39673 CCUG 39693	J. LiPuma D. Speert J. LiPuma D. Speert J. LiPuma J. LiPuma J. LiPuma	CF patient (USA) CF patient (Canada, 1992) CF patient (USA) CF patient (USA, 1997) CF patient (USA) CF patient (USA) CF patient (USA)
<i>Pandoraea</i> sp. R-5199	CCUG 39680, JB1	M. Mergeay	Garden soil
<i>Ralstonia pickettii</i> LMG 5942 ^T	CCUG 3318 ^T	M. Pickett	Patient after tracheotomy (USA)
<i>Ralstonia paucula</i> LMG 3244 ^T	CCUG 12507 ^T	R. Weaver	Human respiratory tract (USA)

and fatty acid methyl esters were prepared, separated and identified using the Microbial Identification System (Microbial ID) as described previously (Vandamme *et al.*, 1992).

RESULTS

Phenotypic characterization

The following characteristics were present in all of the *Pandoraea* strains investigated: catalase activity; growth at 30 and 37 °C; growth on Drigalsky agar; growth in 0.5 and 1.5% NaCl; assimilation of D-gluconate, L-malate and phenylacetate; acid and alkaline phosphatase and leucine arylamidase activity. The following features were absent in all the *Pandoraea* strains investigated: growth in 6.0% NaCl and 10% lactose; hydrolysis of Tween 80 and aesculin; nitrite reduction; denitrification; gelatin liquefaction; growth in O/F medium with adonitol, fructose and xylose; indole production; production of acid or H₂S from TSI agar; assimilation of trehalose, L-arginine, DL-norleucine, L-arabinose, D-mannose, D-mannitol and N-acetylglucosamine; DNase, ornithine decarboxylase, lysine decarboxylase, tryptophanase, C₁₄-lipase,

valine arylamidase, trypsin, chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, N-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase activity. Other features were taxon- or strain-dependent and are shown in Table 2.

None of the isolates tested was able to cause soft rot on onions under the conditions used. All isolates investigated grew on *B. cepacia*-selective medium, except strain R-5199, which showed no growth even after prolonged incubation (96 h). None of the strains was able to degrade PHB under the conditions used.

SDS-PAGE of whole-cell proteins

Reproducibility was checked by preparing protein extracts in duplicate. The correlation level between these patterns was more than 93% (data not shown). After numerical analysis and visual comparison of the profiles, six clusters could be delineated above a correlation level of 85% (Fig. 1). Cluster A was

Table 2. Differential biochemical characteristics

Results are expressed as the percentage of strains investigated that gave a positive reaction; (+) or (–) indicates positive or negative reaction of type strain.

Character	<i>P. norimbergensis</i> (n = 4)	<i>P. pulmonicola</i> (n = 3)	<i>P. pnomenusa</i> (n = 6)	<i>P. apista</i> (n = 11)	<i>P. sputorum</i> (n = 7)	<i>Pandoraea</i> sp. R-5199 (n = 1)
Growth at 42 °C	0	100	100	100	57 (+)	0
Growth on:						
Acetamide	0	0	16 (–)	0	0	0
Cetrimide agar	100	100	66 (–)	100	62 (+)	0
Growth in O/F medium with D-glucose	25 (+)	100	0	0	0	0
Nitrate reduction	0	0	83 (+)	0	14 (+)	100
Growth in:						
3.0% NaCl	25 (–)	0	100	82 (–)	57 (+)	0
4.5% NaCl	0	0	50 (–)	9 (–)	0	0
Assimilation of:						
Caprate	75 (–)	100	100	82 (–)	62 (–)	0
Adipate	0	0	16 (–)	55 (–)	62 (+)	0
DL-Lactate	75 (+)	100	100	100	29 (+)	100
Mannose, mannitol, maltose, N- acetylglucosamine	25 (–)	0	0	0	0	0
Urease activity	75 (–)	0	100	100	14 (–)	100
Oxidase activity	100	100	33 (–)	64 (–)	57 (+)	0
C ₄ -esterase activity	66 (+)*	0	84 (–)	82 (+)	85 (+)	100
C ₈ -ester-lipase activity	100*	0	100	100	100	100
Cysteine arylamidase activity	0*	0	16 (+)	9 (–)	14 (–)	0
Phosphoamidase activity	100*	100	84 (+)	100	100	100

* n = 3.

Fig. 1. Dendrogram derived from the unweighted pair group average linkage of correlation coefficients between the protein patterns of the strains studied. The correlation coefficient is expressed as percentage similarity for convenience.

composed of eight strains. Cluster B comprised five strains. *B. norimbergensis* LMG 18379^T and three other strains (LMG 13019, R-4026 and LMG 16603) constituted cluster C. Three strains formed cluster D. Clusters E and F contained 11 and 5 strains, respectively. Finally, strain R-5620 and reference strains of members of the *B. cepacia* complex, *R. pickettii* and *R. paucula* occupied separate positions in the dendrogram. A selection of protein patterns of representative strains from each cluster is shown in Fig. 2. Visual comparison of the protein patterns indicated that strain R-5620 belongs to cluster B. However, in the numerical analysis of the protein patterns R-5620 occupies a separate position. This is due to significant and reproducible distortions of its protein pattern (Fig. 2), which can have a profound effect on numerical analysis as discussed previously (Vandamme *et al.*, 1997). The same is true for strain R-5199, which

groups in cluster A but has a significantly different protein pattern.

AFLP fingerprinting

Reproducibility was checked by preparing PCR products in duplicate and the similarity between patterns obtained with two PCR products was always higher than 92%. Following numerical analysis, five clusters could be delineated, whilst several strains occupied a separate position (Fig. 3). Cluster I comprised the type strain of *P. norimbergensis* and strains LMG 13019, R-4026 and LMG 16603. Clusters II, III, IV and V were composed of 6, 11, 6 and 3 strains, respectively. Reference strains of members of the *B. cepacia* complex, *R. pickettii* and *R. paucula*, and strain R-5199 occupied separate positions in the dendrogram.

Fig. 2. Electrophoretic protein patterns of a selection of the strains investigated. The molecular mass markers used (lane M) were (from left to right) lysozyme (14.5 kDa), trypsin inhibitor (20 kDa), trypsinogen (24 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), egg albumin (45 kDa), bovine albumin (60 kDa) and β -galactosidase (116 kDa).

16S rRNA gene sequencing

The 16S rRNA sequences of representative strains of the protein electrophoretic and/or AFLP clusters were determined and compared with available 16S rRNA sequences of *B. norimbergensis* LMG 18379^T and strain R-5199 and other representatives of the β -*Proteobacteria*. All 16S rRNA gene sequences determined in the present study were closely related to the sequence of *B. norimbergensis* LMG 18379^T and strain R-5199 (Fig. 4), forming a homogeneous cluster with internal 16S rRNA gene sequence similarities above 96%. Similarity levels towards other taxa belonging to the β -*Proteobacteria* were below 95.3%.

DNA–DNA hybridizations and determination of G + C content

DNA was prepared from strains representing each protein electrophoretic cluster. The DNA–DNA binding values and the G + C content of all strains examined are shown in Table 3. All strains investigated had G + C contents between 61.2 and 64.3 mol%. The hybridization results revealed that there were five groups with internal DNA–DNA binding values higher than 88%. DNA–DNA binding values between representatives of the different groups were between 32 and 45%.

Cellular fatty acid analysis

Both quantitative and qualitative differences in cellular fatty acid composition occur between the different *Pandoraea* species (Table 4). The predominant fatty acids in all strains investigated were 16:0, 17:0 cyclo, 16:0 3-OH, 19:0 cyclo ω 8c, summed feature 3 (comprising 14:0 3-OH, 16:1 iso I, an unidentified fatty acid with equivalent chain length of 10.928, or 12:0

ALDE, or any combination of these fatty acids), summed feature 4 (comprising 16:1 ω 7c or 15 iso 2-OH or both) and summed feature 7 (comprising 18:1 ω 7c, 18:1 ω 9t, or 18:1 ω 12t, or any combination of these fatty acids).

DISCUSSION

We investigated a set of strains, primarily isolated from sputa of CF patients and the environment, which proved difficult to identify in routine diagnostic laboratories. These strains were tentatively grouped by SDS-PAGE of whole-cell proteins and AFLP fingerprinting, and a polyphasic taxonomic approach (including 16S rDNA sequencing, DNA–DNA hybridization, cellular fatty acid analysis and extensive biochemical characterization) was used to clarify their relationships and to determine their phylogenetic position.

Phylogenetic position and structure of the genus *Pandoraea*

Representative strains of the different protein electrophoretic and AFLP clusters (LMG 13019, LMG 18089, LMG 16407^T, LMG 18087^T and LMG 18106^T) formed a single phylogenetic lineage together with *B. norimbergensis* LMG 18379^T and strain R-5199, with internal similarity levels being higher than 96.0% (Fig. 4). The closest related genera are *Burkholderia* and *Ralstonia*, but similarities towards the type species of these genera (*B. cepacia* and *R. pickettii*) are below 93.2% and 92.1%, respectively. Bootstrap analysis indicates that these strains represent a stable clade of species with a phylogenetic position intermediate between the genera *Burkholderia* and *Ralstonia*. The stability of the cluster and the low similarity levels

Fig. 3. Dendrogram derived from the unweighted pair group average linkage of correlation coefficients between the AFLP patterns of the strains studied. The correlation coefficient is expressed as percentage similarity for convenience.

towards the type species of the closest related genera led to the conclusion that these strains represent a novel genus, for which we propose the name *Pandoraea*. Since the type strain of *B. norimbergensis* belongs to this cluster, this species should be transferred to the new genus as *Pandoraea norimbergensis* (Wittke *et al.* 1997) comb. nov.

The dendrograms shown in Figs 1 and 3 suggested that at least five different genomic groups were present within the new genus. Since the similarity levels between the respective rRNA genes of strains belonging to the different clusters were higher than 97%, DNA–DNA hybridizations were performed to

determine their exact degree of genetic relatedness (Stackebrandt & Goebel, 1994). Strains representing all of the AFLP clusters were included in DNA–DNA hybridizations. The results suggested that each AFLP cluster forms one single species. The results also suggested that strains belonging to protein electrophoretic clusters A (except for R-5199), B, C and D form one single species each. Strains LMG 16407^T (cluster E) and R-4527 (cluster F) exhibit high DNA–DNA binding (94%) (as could be expected from the AFLP results: both strains group in cluster III) and can therefore be considered as two electrophoretic types of a single species (Costas, 1992). We conclude that strains from each AFLP cluster con-

Fig. 4. Distance matrix tree showing the phylogenetic position of the genus *Pandoraea* (boxed) within the β -Proteobacteria based on 16S rDNA sequence comparisons. *E. coli* was used as an outgroup in this analysis. The scale bar indicates 10% sequence dissimilarity.

stitute a single species: *P. norimbergensis* (AFLP cluster I), *P. pnomenusa* (cluster II), *P. apista* (cluster III), *P. sputorum* (cluster IV) and *P. pulmonicola* (cluster V). Strain R-5199 occupies a separate position and probably does not belong to any of the described *Pandoraea* species.

Differentiation and identification of *Pandoraea* species

Several biochemical characters are useful to differentiate *Pandoraea* species (Table 2). The most important are growth at 42 °C, growth in O/F medium with D-glucose, growth in 3% NaCl, nitrate reduction and urease, oxidase, C₄-esterase and C₈-ester-lipase activity.

Both quantitative and qualitative differences occur in the fatty acid composition of the different *Pandoraea*

species (see Table 4). In particular, the relative amounts of summed feature 7 and 19:0 cyclo ω 8c vary strongly amongst species. *P. pnomenusa* and *P. apista* can be differentiated from *P. norimbergensis*, *P. pulmonicola* and *P. sputorum* by the presence of significant amounts of 18:1 2-OH. In general however, these differences are small and it seems questionable if they will suffice to identify strains to the species level.

Figs 1 and 2 show that different *Pandoraea* species are characterized by different whole-cell protein profiles. However, the presence of distortions in the patterns, similar to the reported distortions in *Burkholderia* and *Ralstonia* strains (Vandamme *et al.*, 1997; Coenye *et al.*, 1999a; Vandamme *et al.*, 1999), hinder identification of strains based solely on numerical analysis, and strains occasionally may occupy misleading positions in the dendrogram (cf. the position of strain *P. pnomenusa* R-5620). Therefore visual comparison

Table 3. DNA–DNA binding values and G+C content of all strains examined

Mean standard deviation of DNA–DNA hybridization values is approximately 7% (Goris *et al.*, 1998).

Strain	G+C content (mol %)	DNA-binding value (%) with strain:							
		LMG 18379 ^T	LMG 13019	LMG 18106 ^T	LMG 18087 ^T	R-4805	LMG 16407 ^T	R-4527	LMG 18819 ^T
<i>P. norimbergensis</i>									
LMG 18379 ^T	63.2	100							
LMG 13019	62.9	100	100						
<i>P. pulmonicola</i>									
LMG 18106 ^T	61.8	44		100					
<i>P. pnomenusa</i>									
LMG 18087 ^T	64.3	44		42	100				
R-4805	63.0				89	100			
<i>P. apista</i>									
LMG 16407 ^{T*}	61.8	32	33	39	37	34	100		
R-4527 [†]	61.2			35	41		95	100	
<i>P. sputorum</i>									
LMG 18819 ^T	61.9	35		39	45		40	33	100
R-2456	61.8	34	33				36		88

* Protein electrophoretic cluster F.

† Protein electrophoretic cluster E.

Table 4. Fatty acid composition of the strains studied

Results are expressed as mean percentages $\pm$ SD. Those fatty acids for which the mean amount for all taxa was less than 1% are not given.

	12:0	12:0 2-OH	16:0	17:0 cyclo	16:0 2-OH	16:0 3-OH	19:0 cyclo ω 8c	18:1 2-OH	Summed feature 3*	Summed feature 4 [†]	Summed feature 7 [‡]
<i>P. norimbergensis</i> (n=4)	3.2 $\pm$ 0.3	1.1 $\pm$ 0.2	24.8 $\pm$ 0.8	19.8 $\pm$ 2.6	1.0 $\pm$ 0.1	6.1 $\pm$ 0.3	14.9 $\pm$ 4.8	tr	8.2 $\pm$ 0.6	6.3 $\pm$ 2.6	11.2 $\pm$ 3.9
<i>P. pulmonicola</i> (n=3)	2.5 $\pm$ 0.1	1.8 $\pm$ 0.1	26.9 $\pm$ 0.4	17.7 $\pm$ 0.3	2.4 $\pm$ 0.2	7.5 $\pm$ 0.3	17.8 $\pm$ 1.2	tr	8.9 $\pm$ 0.1	4.1 $\pm$ 0.4	6.5 $\pm$ 0.8
<i>P. pnomenusa</i> (n=6)	1.5 $\pm$ 0.1	2.2 $\pm$ 0.2	22.8 $\pm$ 1.3	13.0 $\pm$ 2.1	1.7 $\pm$ 0.4	8.0 $\pm$ 1.5	9.1 $\pm$ 2.2	3.2 $\pm$ 0.7	8.9 $\pm$ 1.0	7.6 $\pm$ 2.0	17.2 $\pm$ 2.5
<i>P. apista</i> (n=11)	1.7 $\pm$ 0.6	2.4 $\pm$ 0.4	23.0 $\pm$ 3.2	16.2 $\pm$ 1.8	1.3 $\pm$ 0.4	7.0 $\pm$ 0.7	11.0 $\pm$ 4.7	2.0 $\pm$ 0.6	8.5 $\pm$ 1.2	7.5 $\pm$ 3.2	15.4 $\pm$ 4.7
<i>P. sputorum</i> (n=7)	2.0 $\pm$ 0.4	2.2 $\pm$ 0.3	26.8 $\pm$ 1.4	15.8 $\pm$ 2.3	1.1 $\pm$ 0.5	5.8 $\pm$ 0.4	9.6 $\pm$ 2.1	tr	7.5 $\pm$ 0.7	6.6 $\pm$ 1.3	17.4 $\pm$ 3.9
<i>Pandoraea</i> sp. R-5199 (n=1)	4.0	ND	20.76	14.43	1.66	4.57	13.74	1.11	6.75	11.06	18.45

ND, Not detected.

tr; trace amount (less than 1%).

* Summed feature 3 comprises 14:0 3-OH, 16:1 iso I, an unidentified fatty acid with equivalent chain length value of 10.928, or 12:0 ALDE, or any combination of these fatty acids.

† Summed feature 4 comprises 16:1 ω 7c or 15 iso 2-OH or both.

‡ Summed feature 7 comprises 18:1 ω 7c, 18:1 ω 9t, or 18:1 ω 12t, or any combination of these fatty acids.

of the protein profiles is necessary to avoid mis-identification.

Studies have shown that AFLP fingerprinting allowed species level identification of strains belonging to the genera *Xanthomonas* (Janssen *et al.*, 1996), *Aeromonas* (Janssen *et al.*, 1996; Huys *et al.*, 1996), *Acinetobacter* (Janssen *et al.*, 1997), *Ralstonia* (Coenye *et al.*, 1999a) and *Burkholderia* (Coenye *et al.*, 1999b) and that the results obtained were generally consistent with DNA–DNA hybridization levels. In the present study, all

Pandoraea species could easily be distinguished by AFLP fingerprinting (Fig. 3) and the results were in agreement with those obtained by DNA–DNA hybridization (Table 3).

Description of *Pandoraea* gen. nov.

Pandoraea (Pan.do.rae'a. N.L. fem. n. *Pandoraea* referring to Pandora's box in Greek mythology, which was the origin of diseases of mankind, and thus to the surprisingly diverse members of this genus).

Cells are Gram-negative, non-sporulating, straight rods of 0.5–0.7 by 1.5–4.0 µm. They occur singly and are motile by means of a single polar flagellum. Catalase activity is present. Growth is observed at 30 and 37 °C. Nitrite is not reduced. No denitrification. No β -galactosidase or DNase activity. No liquefaction of gelatin. No aesculin hydrolysis. No indole production. No hydrolysis of Tween 80. Additional characteristics are listed in the Results section above. The following fatty acid components are present: 12:0, 12:0 2-OH, 16:0, 17:0 cyclo, 16:0 2-OH, 16:0 3-OH, 19:0 cyclo ω 8c, 18:1 2-OH, summed feature 3, summed feature 4 and summed feature 7. The G+C content is between 61.2 and 64.3 mol%. Isolated from human clinical samples (mostly CF patients) and the environment. Does not cause soft-rot on onions. The clinical data that are presently available indicate that at least some of these organisms may cause chronic infection in, and have been transmitted amongst, CF patients. The type species is *Pandoraea apista* sp. nov.

Description of *Pandoraea apista* sp. nov.

Pandoraea apista (a.pis'ta. Gr. adj. *apistos* disloyal, unfaithful, treacherous, referring to the adverse effects of infection for patients).

The description of *P. apista* is the same as that for the genus. Other characteristics are: no growth in O/F medium with glucose or on acetamide; no fluorescence on King's B medium; no nitrate reduction; no assimilation of maltose or sucrose; urease, C_8 -ester lipase, phosphoamidase, amylase and arginine dihydrolase activity present; growth on cetrimide agar; assimilation of citrate, DL-lactate and caprate. The major fatty acids present are 16:0, 17:0 cyclo, 19:0 cyclo ω 8c and summed feature 7. The G+C content is between 61.2 and 61.8 mol%. Isolated from sputum of CF patients. The type strain is LMG 16407^T (=CCUG 38412^T), isolated from sputum of a CF patient in Denmark. The description of the type strain is the same as that given above for the species. Its G+C content is 61.8 mol% and the GenBank accession number for its 16S rDNA sequence is AF139173.

Emended description of *Pandoraea norimbergensis* (Wittke et al. 1997) comb. nov.

Pandoraea norimbergensis [no.rim.ber.gen'sis. M.L. *Norimberga* Nürnberg (Bavaria, Germany); M.L. fem. adj. *norimbergensis* coming from Nürnberg, referring to its place of isolation].

The original description of *P. norimbergensis* was based on data for one strain only. In the present study, we identified three additional isolates as the same species. An emended description of *P. norimbergensis* is given below.

The description of *P. norimbergensis* is the same as that for the genus. Additional characteristics are: no

growth at 42 °C; no fluorescence on King's B medium; no nitrate reduction; no growth on acetamide or in 4.5% NaCl; no assimilation of maltose, adipate or sucrose; no cysteine arylamidase activity present; assimilation of citrate; phosphoamidase activity present; urease activity is strain-dependent. The major fatty acids present are 16:0, 17:0 cyclo, 19:0 cyclo ω 8c and summed feature 7. Isolated from the environment and human clinical samples. The type strain is LMG 18379^T (=CCUG 39188^T). Phenotypic characteristics are the same as described above for the species. In addition, the type strain assimilates D-glucose, arginine dihydrolase activity is present and urease activity is not present. Its G+C content is 63.2 mol% and the GenBank accession number of its 16S rDNA sequence is Y09879. Strain LMG 18379^T was isolated from an oxic water layer above a sulfide-containing sediment in Nürnberg (Germany). Other strains have been isolated from human clinical samples and powdered milk. The GenBank accession number for the 16S rDNA sequence of strain LMG 13019 (=CCUG 38411) is AF139171.

Description of *Pandoraea pulmonicola* sp. nov.

Pandoraea pulmonicola (pul.mo.ni'co.la. L. n. *pulmo* lung; L. suf. *cola* dwelling, occurring in; N.L. n. *pulmonicola* occurring in lungs).

The description of *P. pulmonicola* is the same as that for the genus. Additional characteristics are: no growth on acetamide, or in 3.0 or 4.5% NaCl; no fluorescence on King's B medium; no assimilation of maltose, adipate or sucrose; no nitrate reduction; no amylase, urease, arginine dihydrolase, C_4 -esterase, C_8 -ester lipase or cysteine arylamidase activity present; growth at 42 °C; growth in O/F medium with glucose and on cetrimide agar; assimilation of caprate, citrate, DL-lactate and D-glucose; oxidase and phosphoamidase activity present. The major fatty acids are 16:0, 17:0 cyclo, 19:0 cyclo ω 8c and summed feature 3. Isolated from sputum of CF patients. The type strain is LMG 18106^T (=CCUG 38759^T), isolated from sputum of a CF patient in Canada. The description of the type strain is the same as that given above for the species. Its G+C content is 61.8 mol% and the GenBank accession number for its 16S rDNA sequence is AF139175.

Description of *Pandoraea pnomenusa* sp. nov.

Pandoraea pnomenusa [pno.me.nu'sa. Gr. n. *pnoe* breath, breathing; Gr. v. *meno* to reside, stay, live (fem. part. pres. *menusa*); N.L. part. adj. *pnomenusa* referring to the lung as the niche of these bacteria].

The description of *P. pnomenusa* is the same as that for the genus. Additional characteristics are: no growth in O/F medium with D-glucose; no fluorescence on King's B medium; no assimilation of D-glucose, maltose, sucrose or adipate; growth at 42 °C; as-

Table 5. Characteristics useful for the differentiation of *Pandoraea* species from *R. pickettii*, *R. paucula* and members of the *B. cepacia* complex

Data for *R. pickettii*, *R. paucula* and members of the *B. cepacia* complex were available from previous studies (Vandamme *et al.*, 1999; E. Falsen, unpublished data).

Character	<i>Pandoraea</i> species (<i>n</i> = 32)	<i>R. pickettii</i> (<i>n</i> = 23)	<i>R. paucula</i> (<i>n</i> = 7)	<i>B. cepacia</i> genomovar			<i>B. multivorans</i> (<i>n</i> = 6)	<i>B. vietnamiensis</i> (<i>n</i> = 7)
				I (<i>n</i> = 14)	III (<i>n</i> = 5)	IV (<i>n</i> = 2)		
Catalase activity	+	—	+	—	+	—	—	—
Hydrolysis of Tween 80	—	+	+	+	+	+	+	+
Lysine decarboxylase activity	—	—	—	+	—	+	—	+
Nitrite reduction	—	+	—	—	—	—	—	—
Denitrification	—	+	—	—	—	—	—	—
Gelatin liquefaction	—	—	—	+	+	+	—	—
Aesculin hydrolysis	—	—	—	+	—	—	—	—
β -Galactosidase activity	—	—	—	+	+	—	+	+
Assimilation of:								
Trehalose	—	—	—	+	+	—	+	+
L-Arginine	—	—	—	+	+	+	+	+
L-Arabinose	—	+	—	+	+	—	+	+
Phenylacetate	+	—	+	+	+	+	+	+
Alkaline phosphatase activity	+	—	+	+	+	+	+	+
<i>N</i> -Acetyl- β -glucosidase activity	—	—	—	+	+	—	—	—
β -Glucosidase activity	—	—	—	+	+	—	—	—

simulation of caprate, citrate and DL-lactate; urease activity present. The major fatty acids present are 16:0, 17:0 cyclo and summed feature 7. Isolated from sputum of CF patients. The type strain is LMG 18087^T (= CCUG 38742^T), isolated from sputum of a CF patient in Edinburgh, UK. The description of the type strain is the same as that given above for the species. Its G+C content is 64.3 mol% and the GenBank accession number for its 16S rDNA sequence is AF139174.

Description of *Pandoraea sputorum* sp. nov.

Pandoraea sputorum (spu.to'.rum. L.n. *sputum* spit, sputum; L. gen. pl. n. *sputorum* of sputa).

The description of *P. sputorum* is the same as that for the genus. Additional characteristics are: no growth in O/F medium with glucose or on acetamide; no assimilation of sucrose; no arginine dihydrolase activity present; C₈-ester lipase and phosphoamidase activity present. The major fatty acids present are 16:0, 17:0 cyclo, 19:0 cyclo ω 8c and summed feature 7. Isolated from sputum of CF patients. The type strain is LMG 18819^T (= CCUG 39682^T), isolated

from a CF patient in the USA. Phenotypic characteristics are the same as described above for the species. In addition, the type strain reduces nitrate, produces fluorescence on King's B medium and assimilates maltose and DL-lactate. Its G+C content is 61.9 mol% and the GenBank accession number for its 16S rDNA sequence is AF139176.

Taxonomic position of strain R-5199

Strain R-5199 was isolated from garden soil by Parsons *et al.* (1988) and received special attention due to its ability to utilize biphenyl, 2-chlorobiphenyl, 3-chlorobiphenyl, 4-chlorobiphenyl, *m*-toluate, *p*-toluate, naphthalene, *m*-hydroxybenzoate and diphenylmethane, and due to its ability to transfer chromosomal DNA fragments encoding these characteristics to other strains (Springael *et al.*, 1996). Based on the 16S rDNA sequence, this strain clearly belongs to the genus *Pandoraea* (Fig. 4). However, SDS-PAGE of whole-cell proteins, AFLP fingerprinting and biochemical data show that this strain does not belong to one of the five described species. At this time, we do not propose a formal name for this strain, pending the availability of similar isolates.

Differentiation of members of the genus *Pandoraea* from *R. pickettii*, *R. paucula* and the *B. cepacia* complex

In the routine diagnostic laboratory, strains belonging to the genus *Pandoraea* have been misidentified as *R. pickettii*, *R. paucula* or *B. cepacia*. Using fatty acid analysis, members of the genus *Pandoraea* can easily be distinguished from both *Ralstonia* and *Burkholderia* species by the absence of 14:0 (Vandamme *et al.*, 1997; Coenye *et al.*, 1999a). Furthermore, compared to *Ralstonia* species, *Pandoraea* species have higher percentages of 16:0 3-OH (Coenye *et al.*, 1999a), and differentiation from *Burkholderia* species is possible due to the presence of significant percentages of 12:0 in *Pandoraea* species, whilst this fatty acid has not been detected in *Burkholderia* species (Vandamme *et al.*, 1997). The G+C content of strains from the genus *Pandoraea* (61.2–64.3 mol%) is lower than that of *Ralstonia* species (64–69%; Vandamme *et al.*, 1999) and that of members of the *B. cepacia* complex (67–69%; Vandamme *et al.*, 1997). Biochemical characteristics useful for differentiation of *Pandoraea* species from *R. pickettii*, *R. paucula* and members of the *B. cepacia* complex are given in Table 5. In addition, *Pandoraea* strains can be distinguished from phenotypically similar taxa by SDS-PAGE of whole-cell proteins, AFLP fingerprinting and 16S rDNA sequence determinations.

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