Pseudomonas otitidis sp. nov., isolated from patients with otic infections

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A novel *Pseudomonas* species, for which the name *Pseudomonas otitidis* sp. nov. is proposed, was identified from clinical specimens of infected human ears. Forty-one pseudomonads (34 from patients with acute otitis externa, six from patients with acute otitis media with otorrhoea and one from a patient with chronic suppurative otitis media) were recovered that did not match any known species. On the basis of genetic analyses and biochemical characterization, these isolates were shown to belong to the genus *Pseudomonas*. 16S rRNA gene sequence analysis and DNA–DNA hybridization studies indicated that this novel bacterium is closely related to, but different from, *Pseudomonas aeruginosa*. A description of this species is based on polyphasic studies of 11 clinical isolates. The type strain of *Pseudomonas otitidis* is MCC10330^T (=ATCC BAA-1130^T=DSM 17224^T).

The genus *Pseudomonas* was first described by Migula (1894) and since then members of the genus have been isolated from humans, plants and various environmental habitats, such as soil (Roland & Stroman, 2002; Munsch *et al.*, 2002; Ivanova *et al.*, 2002; Sikorski *et al.*, 2001). The genus *Pseudomonas* was formerly described by Palleroni (1984) as consisting of more than 100 species of phylogenetically unrelated groups of *Proteobacteria*. Palleroni *et al.* (1973), using genetic analysis, specifically rRNA–DNA hybridization, subdivided the genus into five rRNA groups (I–V). Kersters *et al.* (1996) have summarized the changes in the reclassification of the former *Pseudomonas* species, which are now classified into several genera belonging to the *Alphaproteobacteria*, *Betaproteobacteria* or *Gammaproteobacteria*. Species belonging to the genus *Pseudomonas sensu stricto* are now placed in rRNA group I (Palleroni, 1984), which belongs to the *Gammaproteobacteria* (Kersters *et al.*, 1996; Achouak *et al.*, 2000). In the past two decades, polyphasic taxonomic studies, especially those using genetic and molecular techniques, have played a critical role in improving the classification of the pseudomonads (Anzai *et al.*, 2000). Many of the organisms originally described as species of the genus *Pseudomonas* have been reclassified to other genera.

Abbreviation: MIC, minimal inhibitory concentration.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of MCC10330^T is AY953147.

Tables detailing the biochemical and phenotypic characteristics of individual novel strains and the cellular fatty acid composition of the novel strains and related *Pseudomonas* species are available as supplementary material in IJSEM Online.

Novel pseudomonads were recovered from the ears of patients enrolled in anti-infective clinical studies. In total, 41 novel pseudomonads were recovered from ears of patients diagnosed with acute otitis externa ($n=34$), otitis media with otorrhoea drainage through a tympanostomy tube ($n=6$) and chronic suppurative otitis media ($n=1$). A brief description of the source of 11 representative isolates is listed in Table 1. At clinical examination, a swab was used to sample each patient's ears, and the swabs were then immediately placed into transport media. Swabs were removed from transport media and cultured onto various media to obtain viable colonies and then subcultured to obtain a pure culture. Isolated colonies were transferred to trypticase soy broth supplemented with 10 % glycerol and stored in liquid nitrogen. Bacteria were thawed and cultured onto blood agar prior to genetic or phenotypic analyses. DNA–DNA hybridization, comparative 16S rRNA gene sequencing and phenotypic analysis identified these organisms as members of a novel *Pseudomonas* species. The novel pseudomonads were compared with the type strains of *Pseudomonas alcaligenes*, *P. aeruginosa*, *P. citronellolis*, *P. multiresistans*, *P. nitroreducens*, *P. resinovorans* and *P. stutzeri*, which were acquired from the ATCC.

Ribotyping was performed on all 41 strains with the RiboPrinter Microbial Characterization System (DuPont Qualicon) following the manufacturer's recommendations. The novel strains underwent ribotype analyses using a standard ribotyping kit incorporating *EcoRI* restriction endonuclease for species-level identification and *PstI* and *PvuII* endonuclease digestion for strain-level differentiation. *EcoRI* endonuclease digestion of the novel strains produced

Table 1. Sources of *Pseudomonas otitidis* sp. nov. isolates

F, Female; M, male.

Strain	Gender	Age (years)	Diagnosis
MCC09159	F	40	Chronic suppurative otitis media
MCC10330 ^T	M	6	Acute otitis externa
MCC10429	M	13	Acute otitis externa
MCC10744	F	11	Acute otitis externa
MCC11140	M	24	Acute otitis externa
MCC11338	M	60	Acute otitis externa
MCC11683	F	16	Acute otitis externa
MCC12065	M	33	Acute otitis externa
MCC12178	F	15	Acute otitis externa
MCC40150	M	9	Otitis media with otorrhoea drainage through tympanostomy tube
MCC51196	M	3	Otitis media with otorrhoea drainage through tympanostomy tube

a predominant ribotype pattern (39 of 41 strains) with a distinct band at 5·3 kbp; this particular band was shifted to 6 kbp for the two remaining isolates. A representative pattern for the novel taxon is compared with the patterns of the type strains of *P. aeruginosa*, *P. resinovorans*, *P. alcaligenes*, *P. citronellolis* and *P. stutzeri* in Fig. 1. Strain-level discrimination of the new isolates is shown in Fig. 2. The ribotype pattern of the *Pst*I digests revealed a band at 6·9 kbp that was present for every isolate, and the majority of isolates had double bands at approximately 6·5–7·5 kbp. Other bands were present, but no discernible pattern was noted. The ribotype pattern of *Pvu*II digests showed a distinct band at 3·4 kbp that was present for all the isolates. Ribotype patterns of *Pvu*II digestion also produced numerous bands that did not demonstrate any obvious patterns or similarity between isolates.

Bacterial genomic DNA for comparative sequence analysis was extracted using PrepMan UltraSample preparation reagent (Applied Biosystems). Approximately 1451 nt of the 16S rRNA gene was amplified by PCR using the MicroSeq

Full Gene kit (Applied Biosystems). PCR products were sequenced with the MicroSeq Full Gene 16S rRNA gene bacterial sequencing kit (Applied Biosystems). Sequences were determined with a 3100-*Avant* Genetic Analyzer (Applied Biosystems). Phylogenetic analysis was performed using MicroSeq Microbial Identification software version 1.4.1 and BioNumerics software version 3.5 (Applied Maths). Full 16S rRNA gene sequences of *Pseudomonas* species were obtained from GenBank (<http://www.ncbi.nlm.nih.gov/entrez/>). A phylogenetic tree comparing species of *Pseudomonas sensu stricto* that were closely related to the novel taxon was created in BioNumerics by using the neighbour-joining method with a pairwise alignment and no correction factors. Comparative sequence analysis of the 16S rRNA gene revealed that representative strain MCC10330^T is a member of the *Gammaproteobacteria* and that this organism should be included in the genus *Pseudomonas*. Fig. 3, a phylogenetic tree obtained by using the neighbour-joining method, shows the phylogenetic placement of MCC10330^T branching with *P. aeruginosa* (98·6% 16S rRNA gene sequence similarity). The other species most closely related (percentage sequence similarity) to MCC10330^T were *P. alcaligenes* (98·2%), *P. stutzeri* (98·0%), *P. resinovorans* (97·5%), *Pseudomonas pseudoalcaligenes* (97·1%) and *P. citronellolis* (96·9%).

Genomic DNA for spectroscopic DNA–DNA hybridization studies was isolated using a French pressure cell (Thermo Spectronic) and purified by chromatography on hydroxyapatite, as described by Cashion *et al.* (1977). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970) with the modifications suggested by Huß *et al.* (1983) using a Cary 100 Bio UV/Vis spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with *in-situ* temperature probe (Varian). DNA–DNA hybridizations (performed in duplicate) between the new taxon and other related pseudomonads had the following binding values (percentage relatedness): *P. alcaligenes* (55%), *P. citronellolis* (44%),

Fig. 1. Riboprint patterns using *Eco*RI restriction endonuclease comparing representative novel isolate MCC10330^T with related type strains.

Fig. 2. Strain-level discrimination of 11 representative isolates of *Pseudomonas otitidis* sp. nov. using *PstI* and *PvuII* endonuclease digestion.

P. aeruginosa (40 %), *P. nitroreducens* (41 %), *P. resinovorans* (19 %) and *P. stutzeri* (12 %). Given a threshold level of 70 % DNA–DNA relatedness used to delineate a bacterial species, as recommended by Wayne *et al.* (1987), the results indicated that MCC10330^T did not belong to any of the *Pseudomonas* species tested. The DNA G+C content of MCC10330^T was 67.9 mol%.

The 11 representative novel strains were evaluated for phenotypic characteristics and compared with the type strains of *P. aeruginosa*, *P. citronellolis* and *P. stutzeri* (Table 2; results for the individual novel strains are given in Supplementary Table S1 in IJSEM Online). Carbon substrate utilization was determined using the Biolog GN Micro-Plate System and VITEK32 (bioMérieux). The presence of oxidase, catalase and urease activity, production of fluorescent pigments, hydrolysis of Tween 80, aesculin, starch and casein, motility in semisolid media, tolerance of NaCl and minimal and maximal temperature range were all assayed according to standard procedures. Cells of the novel strains were motile, Gram-negative, non-spore-forming rods occurring as single cells. Growth occurred at temperatures of 7–45 °C, but no growth was observed at 4 or 47 °C. Growth occurred on solid media supplemented with 0–4 % NaCl (w/v), but not at higher salinity. Growth was observed in media only at pH 5–10. The majority of the novel strains (10 of 11) failed to produce pyocyanin or

fluorescein, whereas both *P. aeruginosa* and *P. citronellolis* produced these pigments. Each strain had positive reactions for oxidase and catalase, but all were negative for urease. The novel strains were relatively homogeneous in their metabolic characteristics. They differed from the reference strains in utilization of glycerol, D-gluconic acid, itaconic acid, D-fructose, D-mannitol, acetic acid, cytosine, β-phenylethylamine and sebacic acid.

Cellular fatty acid analyses were performed with the MIDI Sherlock Microbial Identification System and a Hewlett Packard gas chromatograph to identify cellular fatty acids qualitatively and quantitatively. The fatty acid compositions of the novel strains and related *Pseudomonas* species are given in Supplementary Table S2 available in IJSEM Online. The major fatty acids detected (percentage of the total cellular fatty acids) from the novel strains were C_{18:1ω7c} (32.9 %), C_{16:0} (26.9 %), C_{12:0} (9.1 %), C_{12:0} 3-OH (4.0 %) and C_{10:0} 3-OH (2.8 %). C_{12:0} 3-OH is consistently found in members of the genus *Pseudomonas sensu stricto* (Oyauzu & Komagata, 1983). The fatty acid compositions of the reference pseudomonads assayed were qualitatively similar to but varied in concentration from those of the novel strains.

Minimal inhibitory concentrations (MIC) were determined using the National Committee for Clinical Laboratory

Fig. 3. Neighbour-joining tree, based on 16S rRNA gene sequence analysis (~1500 bp), showing the phylogenetic relationship of *Pseudomonas otitidis* sp. nov. and other closely related members of the genus *Pseudomonas*. Numbers at nodes are percentages of bootstrap support based on neighbour-joining analyses of 1500 resampled datasets. GenBank accession numbers are given in parentheses. The scale indicates percentage sequence divergence.

Table 2. Phenotypic and biochemical characteristics of strains of *Pseudomonas otitidis* sp. nov. and type strains of related *Pseudomonas* species

For *P. otitidis* strains, the results for the type strain and for the 10 other tested strains are given; for strain-dependent properties, the number of strains showing the majority characteristic/number of strains tested is shown. Results for individual novel strains are available in Supplementary Table S1 in IJSEM Online. Results are scored as: +, positive; –, negative; d, variable result. All strains were positive for growth at 45 °C, catalase and oxidase, motility, hydrolysis of Tween 80 and utilization of glycogen, α -D-glucose, acetic acid, citric acid, formic acid, lactic acid, propionic acid, succinic acid, D-alanine, L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid and L-proline. All strains were negative for growth on 6% (w/v) NaCl agar, indole and aesculin and for utilization of *N*-acetyl-D-galactosamine, adonitol, L-arabinose, i-erythritol, *myo*-inositol, xylitol, α -D-lactose, D-mannose, D-melibiose, D-raffinose, L-rhamnose, turanose and thymidine.

Characteristic	<i>P. otitidis</i>		<i>P. aeruginosa</i>	<i>P. citronellolis</i>	<i>P. stutzeri</i>
	MCC10330 ^T	10 strains	ATCC 10145 ^T	ATCC 13674 ^T	ATCC 17588 ^T
Pyocyanin production	–	– (9/10)	+	+	–
Fluorescein production	–	– (9/10)	+	+	–
Growth at:					
4 °C	–	–	–	–	+
7 °C	–	+	+	–	+
47 °C	–	–	–	–	–
Urease	–	–	d	–	–
Hydrolysis of:					
Gelatin	+	+	+	–	–
Casein	–	–	d	–	d
Growth on NaCl agar:					
4% (w/v)	+	+	+	+	+
5% (w/v)	–	–	d	–	+
Utilization of:					
<i>N</i> -Acetyl-D-glucosamine	–	–	+	–	–
D-Arabitol	–	–	d	–	–
Glycerol	–	–	+	+	+
D-Mannitol	–	–	+	–	+
D-Sorbitol	–	–	–	–	d
D-Fructose	–	–	+	–	+
L-Fucose	–	–	d	–	d
D-Galactose	–	–	+	–	–
Gentiobiose	–	–	–	–	d
Maltose	–	–	–	–	+
Sucrose	–	–	–	–	d
D-Trehalose	–	–	–	d	d
D-Xylose	–	–	–	+	–
D-Galacturonic acid	–	–	+	+	+
D-Gluconic acid	–	–	+	+	+
D-Glucuronic acid	–	–	–	–	+
Itaconic acid	–	–	+	+	+
Glucuronamide	–	–	–	–	+
L-Arginine	+	+	+	+	–
L-Histidine	+	+	d	+	d
L-Isoleucine	+	+	–	–	–
L-Leucine	+	+	d	+	+
L-Ornithine	+	+	+	+	–
L-Phenylalanine	–	–	–	d	–
D-Serine	–	–	d	–	–
L-Serine	+	+	+	+	d
β -Phenylethylamine	+	+	–	–	–
Cytosine	–	–	+	+	+
γ -Aminobutyric acid	+	+	+	+	d
Acetic acid	–	–	+	+	+

Table 2. cont.

Characteristic	<i>P. otitidis</i>		<i>P. aeruginosa</i>	<i>P. citronellolis</i>	<i>P. stutzeri</i>
	MCC10330 ^T	10 strains	ATCC 10145 ^T	ATCC 13674 ^T	ATCC 17588 ^T
Sebacic acid	—	—	+	+	+
Inosine	—	—	+	—	—
Uridine	—	—	d	—	—
Phenylethylamine	+	+	—	+	—
Putrescine	+	+	+	+	d

Standards broth microdilution method (NCCLS, 2003). Microdilution plates (96 wells) contained specific concentrations of antibiotic incorporated into the wells (PML Microbiologicals). Microdilution plates were inoculated with approximately 5×10^5 c.f.u. bacteria ml⁻¹ and incubated overnight at 35 °C. The MIC was defined as the lowest concentration of antibiotic that inhibits growth of an organism as determined by lack of turbidity. The concentrations of antibiotic that inhibited the growth of 50 % (MIC₅₀) or 90 % (MIC₉₀) of the number of novel strains tested were determined. Antibiotic plates were analysed using a 96-well microplate reader (Dynatech Laboratories) at a wavelength of 590 nm and further analysed by visual inspection to determine turbidity. The novel strains exhibited similar antibiotic susceptibility profiles (Table 3). The isolates were susceptible to aminoglycosides (tobramycin, neomycin, gentamicin), fluoroquinolones (ciprofloxacin, ofloxacin, moxifloxacin) and polymyxin B. However, piperacillin, erythromycin, tetracycline, chloramphenicol, trimethoprim and sulfamethoxazole had minimal ability to inhibit growth of the organisms.

Description of *Pseudomonas otitidis* sp. nov.

Pseudomonas otitidis (o.ti'ti.dis. Gr. n. *ous*, *otos* ear; N.L. suff. *-itis*, *-idis* used in names of inflammations; N.L. gen. n. *otitidis* of inflammation of the ear).

Cells are Gram-negative, motile rods. Colonies on trypticase soy agar are circular, concave and non-pigmented. Colonies grown on trypticase soy agar supplemented with 5 % sheep blood are not haemolytic. Growth occurs at 7–45 °C, pH 5–10 and in the presence of up to 4 % NaCl. Colonies typically do not produce fluorescent pigment, and all strains are positive for oxidase and catalase, but negative for urease. Tween 80 and gelatin are hydrolysed, but casein and aesculin are not. Utilizes glycogen, α -D-glucose, acetic acid, citric acid, formic acid, lactic acid, propionic acid, succinic acid, D-alanine, L-alanine, L-arginine, L-asparagine, L-aspartic acid, L-glutamic acid, L-histidine, L-leucine, L-ornithine, L-proline, L-serine, γ -aminobutyric acid, phenylethylamine and putrescine. None of the strains utilizes *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, adonitol, D-arabitol, L-arabinose, i-erythritol, *myo*-inositol, glycerol, D-mannitol, D-sorbitol, xylitol, D-fructose, L-fucose,

Table 3. MIC of various antibiotics for *Pseudomonas otitidis* sp. nov. (*n*=41)

The MIC₅₀ is defined as the concentration of antibiotic that inhibits the growth of 50 % of the organisms tested, whereas the MIC₉₀ is the concentration of antibiotic that inhibits the growth of 90 % of the organisms tested.

Antibiotic	MIC ₅₀ (µg ml ⁻¹)	MIC ₉₀ (µg ml ⁻¹)	MIC range (µg ml ⁻¹)
Tobramycin	0.50	1.0	0.13–1.0
Gentamicin	0.25	1.0	0.13–1.0
Neomycin	2.0	4.0	2.0–8.0
Piperacillin	8.0	4.0	2.0–16
Ciprofloxacin	0.06	0.13	0.03–0.13
Ofloxacin	0.50	1.0	0.25–2.0
Moxifloxacin	1.0	2.0	0.50–2.0
Polymyxin B	0.50	1.0	0.25–2.0
Erythromycin	256	512	256–512
Tetracycline	4.0	8.0	4.0–16
Chloramphenicol	64	128	32.0–> 256
Trimethoprim	32	64	8.0–1024
Sulfamethoxazole	256	> 1024	64–> 1024

D-galactose, gentiobiose, α -D-lactose, maltose, D-mannose, D-melibiose, D-raffinose, L-rhamnose, sucrose, D-trehalose, turanose, D-xylose, D-galacturonic acid, D-gluconic acid, D-glucuronic acid, itaconic acid, glucuronamide, L-phenylalanine, D-serine, inosine, uridine, thymidine, cytosine or sebacic acid. The predominant cellular fatty acids are C_{18:1} ω 7c, C_{16:0}, C_{12:0}, C_{12:0} 3-OH and C_{10:0} 3-OH. Susceptible to aminoglycosides, fluoroquinolones and polymyxin B, but resistant to piperacillin, erythromycin, tetracycline, chloramphenicol, trimethoprim and sulfamethoxazole. The DNA G + C content of the type strain is 67.9 mol%.

The type strain, MCC10330^T (= ATCC BAA-1130^T = DSM 17224^T), and other strains were isolated from the ears of patients with acute otitis externa, acute otitis media with otorrhoea and chronic suppurative otitis media.

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