Pseudomonas simiae sp. nov., isolated from clinical specimens from monkeys (*Callithrix geoffroyi*)

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An unusual Gram-negative, catalase- and oxidase-positive, rod-shaped bacterium isolated from different clinical samples from two monkeys (*Callithrix geoffroyi*) was characterized by phenotypic and molecular genetic methods. The micro-organism was tentatively identified as a *Pseudomonas* species on the basis of the results of cellular morphological and biochemical tests. Fatty acid studies confirmed this generic placement and comparative 16S rRNA gene sequencing studies demonstrated that the unknown isolates were phylogenetically closely related to each other (100 % sequence similarity) and were part of the '*Pseudomonas fluorescens* intragenetic cluster'. The novel bacterium, however, was distinguished from other phylogenetically related species of *Pseudomonas* by DNA–DNA hybridization studies and biochemical tests. On the basis of both phenotypic and phylogenetic findings, it is proposed that the novel *Pseudomonas* isolates are classified as *Pseudomonas simiae* sp. nov. The type strain of *P. simiae* is OLi^T (=CCUG 50988^T = CECT 7078^T).

Pseudomonads are widely distributed in agricultural soils, in freshwater and marine environments and in plants and animals. The genus *Pseudomonas* includes species that can be pathogenic for humans and animals (Palleroni, 1984, 1993). *Pseudomonas aeruginosa* is currently the most significant pathogenic species in the genus *Pseudomonas*. This micro-organism has been frequently associated with different human and animal infections (Lausen *et al.*, 1986; Coalson *et al.*, 1988; Las Heras *et al.*, 1999; Daly *et al.*, 1999; Poiriel *et al.*, 2004). Other *Pseudomonas* species have also been involved in human infections, such as *Pseudomonas stutzeri* (Jiraskova & Rozsival, 1998; Puzenat *et al.*, 2004), *Pseudomonas fluorescens* (Smith *et al.*, 2002) and *Pseudomonas putida* (Franzetti *et al.*, 1992; Lombardi *et al.*, 2002). However, animal infections caused by *Pseudomonas* species other than *P. aeruginosa* are often not reported. In this work, we have used phenotypic and molecular genetic methodologies to facilitate the characterization of some *Pseudomonas*-like organisms recovered from clinical samples from

monkeys (*Callithrix geoffroyi*). On the basis of our findings, we propose a novel *Pseudomonas* species that is taxonomically affiliated to the '*P. fluorescens* intragenetic cluster' described by Anzai *et al.* (2000).

A juvenile female monkey (*C. geoffroyi*) and its offspring were submitted to the necropsy service of the Veterinary School Hospital in Madrid, Spain. These animals were housed in the same cage at the submitting facility, a Primate Conservation Centre, and were separated from the rest of the animals. The female monkey was found dead 2 days prior to the death of its offspring. Neither of these two animals had shown clinical signs of disease during the 24 h preceding their deaths. Glomerulonephritis with secondary uraemic pneumonitis and acute bronchointerstitial pneumonia were the clinical signs observed after post-mortem examination of the female monkey and the offspring, respectively. Gram-stained sections of the lungs of the offspring revealed the presence of Gram-negative rods inside the pulmonary alveoli.

Samples from the lungs, liver and brain of the female monkey and its offspring were taken for microbiological

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CCUG 50988^T is AJ936933.

analysis (under aseptic conditions to avoid environmental contamination) and kept under refrigeration until they were processed in the laboratory. Tissue samples were surface-plated on Columbia blood agar and MacConkey agar (bioMérieux) and incubated aerobically and under anaerobic conditions for 48 h at 37 °C. A rod-shaped organism was isolated in pure culture from the lungs, liver and brain of the offspring (isolates OLu, OB and OLi^T) and from the liver and brain of the female monkey (isolates MLi and MB). Oxidase activity was tested with oxidase test sticks (Oxoid). The isolates were cultured on tryptic soy agar (bioMérieux). Motility was observed by means of the hanging drop method and in semi-solid media. The temperature range for growth was determined after cultivation of the isolates in tryptic soy broth (bioMérieux) for 24 h at 30, 37 and 42 °C and for 3 days at 10 and 4 °C. The ability of the isolates to produce a fluorescent pigment was tested on King's B medium (King *et al.*, 1954). Growth in 6.5 % NaCl (w/v) was also examined. Biochemical identification was achieved using the commercial API 20 NE and API ZYM systems (bioMérieux) and Biolog GN Microplates (Biolog) according to the manufacturers' instructions. The clinical isolates were molecularly characterized by PFGE according to the specifications of Blanco *et al.* (2002), with the *Xba*I (Promega) and *Spe*I (MBI Fermentans) restriction endonucleases.

Phylogenetic characterization was performed using 16S rRNA gene sequencing, as described previously (Collins *et al.*, 1999). A large fragment (approx. 1450 bases) of the 16S rRNA gene of two isolates (MLi and OLi^T) and 1000 nt from the other three isolates (MB, OB, OLu) were amplified by using a PCR and were directly sequenced using a *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems) and an automatic DNA sequencer (model 373A; Applied Biosystems). The closest described relatives of the novel isolates were determined by performing searches of GenBank using the FASTA program (Pearson, 1994). These closely related sequences were retrieved from GenBank and aligned with the newly determined sequences using the DNATools program (Rasmussen, 1995). Phylogenetic trees were constructed according to two different methods: a neighbour-joining algorithm (Saitou & Nei, 1987), performed with the programs DNATools and TREEVIEW (Page, 1996), and a maximum-likelihood analysis obtained by using the PHYLIP package (version 3.57; Felsenstein, 1993). The stability of the groupings was estimated by bootstrap analysis (1000 replications). Fatty acid methyl esters were prepared and analysed as described by Kämpfer & Kroppenstedt (1996).

The genotypic relatedness of two clinical isolates (OLi^T and MLi) and between one isolate (OLi^T) and *Pseudomonas poae* DSM 14936^T, *Pseudomonas trivialis* DSM 14937^T and *Pseudomonas extremorientalis* KMM 3447^T were also assessed by means of DNA–DNA hybridization. DNA was extracted and purified by the method of Marmur (1961). DNA–DNA hybridization was studied using the competition procedure of the membrane method of Johnson (1994),

described in detail by Arahall *et al.* (2001). The hybridization experiments were carried out under optimal conditions, at a temperature of 49.5 °C, which is within the limits of validity for the filter method (De Ley & Tijtgaat, 1970). The percentage hybridization was calculated as described by Johnson (1994). Three independent determinations were carried out for each experiment and the results reported are mean values. The G + C content of the DNA was determined from the mid-point value (T_m) of the thermal denaturation profile (Marmur & Doty, 1962) obtained with a Perkin-Elmer UV-Vis Lambda 20 spectrophotometer at 260 nm; this instrument was programmed for temperature increases of 1.0 °C min⁻¹ by using a Peltier temperature programmer (Perkin-Elmer). The T_m value was determined in 0.1× SSC buffer (0.15 M NaCl buffered with 0.015 M trisodium citrate, pH 7.0) by using a graphic method described by Ferragut & Leclerc (1976) and the G + C content was calculated from this temperature by using the equation of Owen & Hill (1979). The T_m value of reference DNA from *Escherichia coli* NCTC 9001 was taken as 74.6 °C in 0.1× SSC (Owen & Pitcher, 1985).

The five isolates were Gram-negative, non-spore-forming rods. The cells were catalase- and oxidase-positive, strictly aerobic and motile. They were slightly β -haemolytic. All of the isolates grew on MacConkey agar. Growth between 4 and 37 °C was observed, but the isolates did not grow at 42 °C. All five isolates were able to grow in 6.5 % NaCl. On tryptic soy agar, a yellow pigment was observed and a fluorescent pigment was produced on King's B medium. The isolates displayed identical phenotypic profiles in the commercial API 20 NE system. All of the isolates reduced nitrate and nitrite, hydrolysed gelatin and gave a positive reaction for arginine dihydrolase. None of the isolates produced indole, β -galactosidase or acid from glucose, and all failed to hydrolyse urea and aesculin. Caprate, L-malate and citrate were assimilated, but *N*-acetyl-D-glucosamine, maltose, adipate and phenylacetate were not. In the API ZYM system, positive reactions for esterase C4, ester lipase C8, leucine arylamidase and acid phosphatase and a weak reaction for valine arylamidase were detected. All other tests were negative. The isolates were characterized further using the Biolog identification system. The results of the Biolog GN assay are indicated in the species description. In terms of their overall cellular morphology and biochemical characteristics, the novel isolates somewhat resembled members of the family *Pseudomonadaceae*. To clarify the association between the novel monkey isolates and the family *Pseudomonadaceae*, one representative strain, OLi^T, was subjected to cellular fatty acid analysis. The profile of strain OLi^T was characterized by the presence of C_{10:0} 3-OH and C_{12:0} 3-OH fatty acids, which is consistent with their classification as *sensu stricto* pseudomonads (Oyaizu & Komagata, 1983). The major fatty acids of the strain were C_{16:0}, C_{16:1} ω 7c, C_{18:1} ω 7c and C_{17:0} cyclo (mean contents 30.3, 27.0, 12.3 and 11.7 %, respectively). The isolate also contained moderate or small amounts of saturated (C_{12:0}, C_{12:0} 2-OH, C_{14:0}, C_{15:0}, C_{17:0}, C_{19:0} ω 8c cyclo), unsaturated

(C_{18:2}ω6,9c, C_{16:1}ω7c DMA) and branched (iso-C_{15:0} 3-OH) fatty acids.

Three different assays confirmed that the DNA G+C contents of isolates OLi^T and MLi were 49.7 and 52.3 mol%, respectively; these percentages being lower than those associated with members of the genus *Pseudomonas* (58–70 mol%; Palleroni, 1984).

To determine the phylogenetic position of the novel isolates, their 16S rRNA gene sequences were analysed. The five isolates all had the same nucleotide sequence (100 % similarity). Sequence database searches (GenBank and Ribosomal Database Project libraries) revealed that the novel isolates were phylogenetically most closely related to members of the genus *Pseudomonas* (results not shown). Phylogenetic trees obtained using the neighbour-joining (Fig. 1) and maximum-likelihood (data not shown) methods revealed a clear affiliation of the novel isolates (as exemplified by strain OLi^T) with the '*P. fluorescens* group' of Anzai *et al.* (2000) and placed the novel strain on a

separate branch within this intrageneric cluster containing *P. fluorescens* DSM 50090^T (GenBank accession number Z76662). Strain OLi^T displayed the closest sequence similarity with *P. poae* (99.8 %; AJ492829), *P. trivialis* (99.7 %; AJ492831) and *P. extremorientalis* (99.5 %; AJ492831). The other species most closely related (in terms of percentage sequence similarity) to strain OLi^T were *Pseudomonas constantinii* (99.5 %; AF374472), *Pseudomonas tolaasii* (99.3 %; Z76670) and *Pseudomonas palleroniana* (99 %; AY091527). Although bootstrap resampling analysis shows that the association between strain OLi^T and these species is not statistically significant, the branching position of this novel strain within this clade was relatively stable according to the two tree-making algorithms used in this study. The topology of the tree obtained by using the neighbour-joining method (Fig. 1) indicates that strain OLi^T forms a separate branch together with *P. poae* and *P. trivialis*, but it was positioned with *P. extremorientalis* in the maximum-likelihood tree. Differences between strain OLi^T and these species in

Fig. 1. Neighbour-joining unrooted tree, based on 16S rRNA gene sequence comparisons, showing the phylogenetic relationships of *Pseudomonas simiae* sp. nov. OLi^T. Bootstrap values (expressed as percentages of 1000 replications) are given at the branching points. Solid circles indicate that the corresponding nodes (groupings) are also recovered in maximum-likelihood trees. Bar, 1 % sequence divergence.

terms of some of the biochemical characteristics and the phylogenetic position based on 16S rRNA gene sequence analysis suggested that the isolates obtained from the monkey clinical samples represent a novel species.

16S rRNA gene sequence divergence values greater than 3% are considered to represent a threshold for defining novel bacterial species (Stackebrandt & Goebel, 1994). Nevertheless, some novel species described as belonging to the genus *Pseudomonas* have exhibited 16S rRNA gene sequences that were more than 99% similar to closely related validated species (Behrendt *et al.*, 2003), indicating that, within the genus *Pseudomonas*, species delineation cannot be based exclusively on 16S rRNA gene sequence similarities. DNA–DNA hybridization experiments are considered the key method for determining the taxonomic position at species level, with DNA–DNA hybridization values of at least 70% being the threshold for species delineation (Wayne *et al.*, 1987). For DNA–DNA hybridization studies, two of the novel clinical isolates (OLi^T and MLi) and some closely related species of the genus *Pseudomonas* were selected on the basis of phenotypic and 16S rRNA gene sequence analyses. When the two clinical isolates were subjected to a chromosomal DNA–DNA hybridization study, they showed 99% DNA–DNA relatedness with each other, demonstrating that they are members of the same species. The DNA–DNA reassociation values between the clinical strain OLi^T and *P. poae* DSM 14936^T, *P. trivialis* DSM 14937^T and *P. extremorientalis* KMM 3447^T were 23, 2 and 30%, respectively, indicating a separate species status for strain OLi^T (Wayne *et al.*, 1987). It is clear from the polyphasic taxonomic study that the five clinical isolates should be considered as representing a distinct species within the genus *Pseudomonas*, for which the name *Pseudomonas simiae* sp. nov. is proposed. Furthermore, the novel bacterium is also phenotypically very different from all recognized *Pseudomonas* species. In particular, the novel bacterium can be readily distinguished from other phylogenetically closely related species (Table 1).

All of the novel clinical isolates displayed undistinguishable macrorestriction patterns in PFGE analyses with both enzymes, indicating that they are probably clonal in origin. Only the isolate from the lung of the monkey offspring was recovered in pure culture; this, together with the histopathological findings, is strongly indicative of the clinical significance of *Pseudomonas simiae* sp. nov. as being responsible for the pneumonic infection observed in the animal. This association could not be established unambiguously with respect to the female monkey, making it difficult, therefore, to determine the potential pathogenic role of *P. simiae* in monkeys. The formal description of this novel species will facilitate its identification in veterinary clinical laboratories, thereby permitting the future evaluation of its distribution, clinical prevalence and possible association with disease. In this sense, *P. simiae* can be easily differentiated from other *Pseudomonas* species (e.g. *P. aeruginosa* or *P. fluorescens*) commonly isolated from

Table 1. Characteristics useful in differentiating *Pseudomonas simiae* sp. nov. from the closest phylogenetic relatives

Taxa: 1, *Pseudomonas simiae* sp. nov. (five strains); 2, *P. poae*; 3, *P. trivialis*; 4, *P. extremorientalis*; 5, *P. constantinii*; 6, *Pseudomonas veronii*; 7, *Pseudomonas rhodesiae*; 8, *P. palleroniana*; 9, *P. tolaasii*; 10, *Pseudomonas marginalis*; 11, *Pseudomonas grimontii*. Data for *Pseudomonas* species were obtained from Elomari *et al.* (1996), Dabboussi *et al.* (1999, 2002), Baïda *et al.* (2002), Ivanova *et al.* (2002), Munsch *et al.* (2002), Gardan *et al.* (2002) and Behrendt *et al.* (2003). *P. poae* and *P. trivialis* can be distinguished from *P. simiae* by their ability to assimilate glucuronamide, but not α -cyclodextrin or dextrin and a negative reaction in tests for arginine dihydrolase. *P. extremorientalis* can be distinguished from *P. simiae* by the ability to assimilate glucuronamide, but not α -cyclodextrin. +, Positive reaction; –, negative reaction; V, variable reaction; ND, not determined.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Nitrate reduction	+	–	–	+	–	+	–*	–	–	+	+
Aesculin hydrolysis	–	+	+	ND	–	+	ND	–	–	+	ND
Gelatinase	+	V	V	–	+	V	–	+	+	+	ND
Assimilation of:											
Sucrose	–	+	–	+	V	+	–†	+	+	‡	+
Maltose	–	–	–	+	–	+	–	ND	+	+	–
D-Trehalose	–	+	+	+	+	V	+	+	+	+	+
α -D-Glucose	+	V	+	–	+	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	+	+	ND	–	+	+
Lactose	–	–	–	–	–	–	–	ND	+	+	–
Melibiose	–	–	–	–	–	–	–	–	+	+	–
L-Rhamnose	–	–	V	–	–	–	–	–	+	V	+
D-Mannitol	+	+	+	+	+	+	–†	ND	–	+	+
L-Phenylalanine	–	–	–	–	ND	+	+	+	+	+	ND
N-Acetyl-D-glucosamine	–	–	V	+	+	+	+	ND	ND	+	+
i-Erythritol	+	–	–	–	ND	+	–	+	+	V	+
D-Xylitol	–	–	–	+	+	–	–	–	+	V	–
Adonitol	+	–	–	–	+	–	–	–	–	–	–
myo-Inositol	+	+	+	–	+	+	+	ND	+	+	+
Itaconic acid	–	+	+	+	+	+	–	+	+	+	+
Putrescine	–	+	V	–	+	ND	ND	ND	ND	+	+
L-Ornithine	+	V	V	–	ND	V	–	+	ND	V	ND

*Positive reaction according to Dabboussi *et al.* (1999).

†Positive reaction according to Elomari *et al.* (1996).

‡Negative reaction according to Gardan *et al.* (2002).

animals with infections by applying several biochemical tests (Baïda *et al.*, 2002; Catara *et al.*, 2002; Ivanova *et al.*, 2002).

Description of *Pseudomonas simiae* sp. nov.

Pseudomonas simiae (si' mi. ae. L. gen. n. *simiae* of an ape, of a monkey).

Gram-negative, catalase- and oxidase-positive, strictly aerobic and motile rods approximately 1–1.5 μ m in size. Cells produce a yellow pigment on tryptic soy agar. Colonies

on Columbia blood agar are circular, non-pigmented and slightly haemolytic. Temperature range for growth is 4–37 °C; optimal growth occurs at 30 °C. Growth occurs in the presence of 6.5% NaCl. Fluorescent pigment is produced on King's medium B. The isolates reduce nitrate and hydrolyse gelatin. None of the isolates produces acid from glucose, acetoin or β -galactosidase. Reaction for arginine dihydrolase is positive. Urea and aesculin are not hydrolysed. The following carbon substrates are assimilated: α -cyclodextrin, dextrin, glycogen, Tweens 40 and 80, adonitol, L-arabinose, D-arabitol, D-erythritol, D-fructose, D-galactose, α -D-glucose, *myo*-inositol, D-mannitol, D-mannose, D-psicose, D-sorbitol, methyl pyruvate, mono-methyl succinate, acetic acid, *cis*-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, α -hydroxybutyric acid, β -hydroxybutyric acid, *p*-hydroxyphenylacetic acid, α -ketobutyric acid, α -ketoglutaric acid, α -ketovaleric acid, DL-lactic acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, succinic acid, bromosuccinic acid, succinamic acid, L-alaninamide, D-alanine, L-alanine, L-alanyl glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-proline, L-pyrogutamic acid, L-serine, L-threonine, DL-carnitine, γ -aminobutyric acid, urocanic acid, inosine, uridine, 2-aminoethanol, glycerol and DL- α -glycerol phosphate. None of the isolates assimilated *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, D-cellobiose, L-fucose, gentiobiose, α -D-lactose, lactulose, maltose, D-melibiose, methyl β -D-glucoside, D-raffinose, L-rhamnose, sucrose, D-trehalose, turanose, xylitol, γ -hydroxybutyric acid, itaconic acid, sebacic acid, glucuronamide, glycyl L-aspartic acid, L-phenylalanine, D-serine, thymidine, phenylethylamine, putrescine, 2,3-butanediol, glucose 1-phosphate or glucose 6-phosphate. Esterase (C4), ester lipase (C8), leucine arylamidase and acid phosphatase activities, and a weak reaction for valine arylamidase are detected. Alkaline phosphatase, lipase, cystine arylamidase, trypsin, chymotrypsin, phosphoamidase, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosamidase, α -mannosidase and α -fucosidase are not produced. The major fatty acids are C_{16:0}, C_{16:1} ω 7c, C_{18:1} ω 7c and C_{17:0} cyclo. The DNA G+C content of strain OLi^T is 49.7 mol% (*T*_m).

The type strain, OLi^T (=CCUG 50988^T=CECT 7078^T), was isolated from the liver of a monkey with acute bronchopneumonia and bacteraemia.

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