NOTE

Reclassification of [*Pseudomonas*] *doudoroffii* (Baumann *et al.* 1983) into the genus *Oceanomonas* gen. nov. as *Oceanomonas doudoroffii* comb. nov., and description of a phenol-degrading bacterium from estuarine water as *Oceanomonas baumannii* sp. nov.

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A bacterium, isolate GB6^T, capable of degrading phenol in the presence of elevated salinity was isolated from the estuary of the River Wear, UK. The bacterium was subjected to biochemical and molecular analysis to determine its taxonomic status. These studies indicated that the bacterium was a distinct species closely related to [*Pseudomonas*] *doudoroffii*. However, the phylogenetic analysis indicated that [*Pseudomonas*] *doudoroffii* was misclassified, as noted previously. Analysis of the characteristics of isolate GB6^T and the type strain of [*Pseudomonas*] *doudoroffii* confirmed that these bacteria belonged to the same novel genus, which we have named *Oceanomonas* gen. nov. The type strain of *Oceanomonas doudoroffii* (Baumann *et al.* 1983) comb. nov. is ATCC 27123^T (= DSM 7028^T). The DNA–DNA homology between isolate GB6^T and [*Pseudomonas*] *doudoroffii* is low and phenotypic differences between the two organisms are evident. Isolate GB6^T (= ATCC 700832^T = NCIMB 13685^T) is therefore proposed as the type strain of a new species, *Oceanomonas baumannii* sp. nov.

Keywords: [*Pseudomonas*] *doudoroffii*, 16S rDNA, *Oceanomonas doudoroffii*

In our search for organisms capable of degrading phenol, we have isolated a bacterium, GB6^T, that we identify herein as a new species closely related to [*Pseudomonas*] *doudoroffii*. [*Pseudomonas*] *doudoroffii* was originally defined to accommodate marine motile, Gram-negative rods (Baumann *et al.*, 1972, 1983), although its taxonomic status has been in question since extensive revisions were made to the family *Pseudomonadaceae* (De Vos *et al.*, 1989; Kersters *et al.*, 1996). The taxonomic reorganization of the pseudomonads has employed a variety of analytical techniques, including phenotypic studies (Stanier *et al.*, 1966), numerical taxonomy (Sneath *et al.*, 1981) and nucleic acid similarity studies (Palleroni *et al.*, 1973). The latter work provided the most important

contribution, as it divided the pseudomonads into five distantly related groups based on rRNA–DNA hybridizations. This approach ultimately led to the recognition that many *Pseudomonas* species were more appropriately classified in other genera. Indeed, the current genus *Pseudomonas* is restricted to species belonging to rRNA group I and the organisms in the remaining four rRNA groups have been reclassified into other genera (Kersters *et al.*, 1996). Despite these revisions, the status of [*Pseudomonas*] *doudoroffii* remains unclear. De Vos *et al.* (1989) suggested that this bacterium was not a member of the genus *Pseudomonas* and was more likely to belong to the *Aeromonadaceae*, but were unable to demonstrate whether this taxon deserved separate generic rank. Here, we demonstrate that [*Pseudomonas*] *doudoroffii* and the organism GB6^T isolated during our studies belong to a genus distinct from both *Pseudomonas* and *Aeromonas*. We therefore propose that [*Pseudomonas*] *doudoroffii* be assigned to the novel genus

Abbreviations: FAME, fatty acid methyl ester; MSM, minimal salts medium.

The GenBank accession number for the 16S rDNA sequence of *Oceanomonas baumannii* is AF168367.

Oceanomonas as *Oceanomonas doudoroffii* comb. nov., with the former [*Pseudomonas*] *doudoroffii* type strain (ATCC 27123^T) as the type strain. In addition, due to the low DNA–DNA homology and phenotypic differences between [*Pseudomonas*] *doudoroffii* and isolate GB6^T, we propose that this strain be assigned to a novel species *Oceanomonas baumannii* with GB6^T (= ATCC 700832^T = NCIMB 13685^T) as the type strain.

Isolation of a phenol-degrading bacterium

Strain GB6^T was isolated from estuarine mud collected from the mouth of the River Wear, Sunderland, UK. Baffled flasks (500 ml) containing 200 ml minimal salts medium (MSM; Hartmans *et al.*, 1989) plus 5% NaCl (w/v) with 4 mM phenol and 50 µg cycloheximide ml⁻¹ at pH 7.2 were inoculated with 5 ml samples of mud. The flasks were incubated at 25 °C in an orbital shaker at 150 r.p.m. for 7 d. Subsequently, 1 ml of the enrichment broth was inoculated into 200 ml fresh medium. The second enrichment broth was then streaked onto plates containing minimal medium plus 1% NaCl (w/v), 4 mM phenol and Oxoid no. 1 bacteriological agar (1.5% w/v). Single colonies were picked and inoculated into minimal medium containing 5% NaCl (w/v) and 4 mM phenol. Isolate GB6^T was chosen for more detailed study as it demonstrated the most rapid growth on phenol. The bacterium was a short, Gram-negative rod (2 × 1 µm) with up to four polar flagella.

Molecular methods and phylogenetic analysis

DNA was prepared from 1 ml of late exponential culture using a QIAamp tissue kit (Qiagen). The 16S rDNA was amplified by PCR using recombinant *Taq* polymerase (Life Technologies) and a pair of primers designed from *Escherichia coli* rDNA, 8-27F (5'-AG-AGTTTGATCCTGGCTCAG-3') and 1509-1491R (5'-GGHTACCTTGTTACGACTT-3') (Weisburg *et al.*, 1991). The conditions for thermal cycling were denaturation of target DNA at 95 °C for 3 min followed by 35 cycles of denaturation at 95 °C for 2 min, primer annealing at the following range of temperatures for 1 min (35, 38, 42 and 46 °C) and primer extension at 72 °C for 4 min. At the end of cycling, the reaction mixture was held at 72 °C for 20 min and cooled. Reaction conditions per tube were as follows: 0.5 µM of each primer, 1 µg template DNA, 2.5 U *Taq* polymerase, 1 × PCR buffer, 0.2 mM of each dNTP and MgCl₂ at 1, 2, 3 or 4 mM. Ten microlitres of mineral oil was added to each tube to prevent evaporation.

The 1.5 kb amplified 16S rDNA product was purified using a PCR purification kit (Qiagen), ligated into pGEM-T EASY (Promega) and transformed into *E. coli* XL10 GOLD (Stratagene). Recombinants were detected by blue–white selection and subsequent plasmids were extracted using a QIAprep Spin Miniprep kit (Qiagen). Automated sequencing was

carried out on an Oligo 1000M DNA Synthesizer (Central Facility for Molecular Biology, University of Newcastle-upon-Tyne, UK). The following primers, designed for this study, were used for the sequencing of both strands of DNA: forward primers 5'-GTA-TCACCCGCTGGCAAAA-3' and 5'-GCCTCGC-CACTGGTATTCTTCC-3' and reverse primers 5'-CTACGGGAGGCAGCAGTGG-3' and 5'-GGA-GCAAACAGGATTAGATACCC-3'.

The rRNA sequence obtained (1296 bases) was aligned manually using the CLUSTAL method in the MEGALIGN program (DNASTar) with reference bacterial 16S rDNA gene sequences taken from the EMBL database using the BLAST search tool (NCBI). Positions where gaps and unresolved bases made alignment uncertain were omitted, resulting in sequences of 1130 bases. Programs used for phylogenetic analysis included SEQBOOT, DNADIST, NEIGHBOR, CONSENSE (PHYLIP 3.5c; Felsenstein, 1993) and TREEVIEW (Page, 1996).

The unrooted phylogenetic dendrogram generated indicated that the similarity between isolate GB6^T and [*Pseudomonas*] *doudoroffii* 16S rDNA was 98.2% (Fig. 1). We compared the 16S rDNA sequence with two previously deposited sequences for [*Pseudomonas*] *doudoroffii* in the EMBL database (GenBank accession numbers AB021371 and AB019390) using the BLAST search tool. When these sequences were compared with each other and the gaps and unresolved bases were excised, the sequences were identical except for one base pair. In turn, these sequences shared 98.2% identity with the sequence of GB6^T, which indicates that these organisms are closely related and potentially strains of the same species.

DNA–DNA hybridization

DNA–DNA hybridization was carried out by the identification service of the DSMZ (Braunschweig, Germany) as described by De Ley *et al.* (1970), with the modifications described by Huss *et al.* (1983) and Escara & Hutton (1980), using a Gilford System model 2600 spectrometer equipped with a Gilford model 2527-R thermoprogrammer and plotter. Renaturation rates were computed with the TRANSFER.BAS program of Jahnke (1992). The relatedness between GB6^T and [*Pseudomonas*] *doudoroffii* was found to be only 38.6%, indicating that the two organisms were in fact two different species, despite the high 16S rDNA identity between the two (Wayne *et al.*, 1987).

Phenotypic analyses

In order to clarify further the relationship between GB6^T and the type strain of [*Pseudomonas*] *doudoroffii*, DSM 7028^T (= ATCC 27123^T), we undertook phenotypic and chemotaxonomic studies. The API20 NE test kit (bioMérieux) was utilized for phenotypic characterization. Oxidase activity was determined by monitoring the oxidation of Kovacs reagent on filter

Fig. 1. Unrooted phylogenetic dendrogram based upon the 16S rDNA sequence of *Oceanomonas baumannii* GB6^T compared with the 15 most closely related organisms taken from the BLAST and EMBL services. Produced using SEQBOOT, DNADIST (Jukes and Cantor), NEIGHBOR (neighbour-joining) and CONSENSE (programs in the PHYLIP package). The K_{nuc} scale bar (nucleotide substitutions per 100 nucleotides) was generated by the TREEVIEW program (Page, 1996). Bootstrap values were generated by CONSENSE from 1000 replicate trees. *Vibrio parahaemolyticus* was used as the outgroup.

paper (Singleton & Sainsbury, 1995). Catalase activity was detected by the production of bubbles after the addition of 3 % hydrogen peroxide onto fresh colonies (Singleton & Sainsbury, 1995). Other substrates not available on the API20 NE kit were substituted for phenol at concentrations of 4 mM. The results of the biochemical tests indicated that isolate GB6^T was obligately aerobic, catalase- and oxidase-positive and could utilize malate, succinate, citrate and galactose as growth substrate. The profile for assimilation of substrates was very similar to that of [*Pseudomonas*] *doudoroffii* DSM 7028^T (Table 1), except that GB6^T was distinct in being capable of utilizing galactose and glycerol. *Ortho*-cleavage of catechol was determined by spraying colonies with 5 % (v/v) catechol solution, which resulted in a brown coloration, indicating that phenol was degraded via the *ortho*-pathway.

Chemotaxonomic lipid analyses

Total lipids were extracted with chloroform and methanol by the method of Bligh and Dyer (Kates, 1986). Individual lipids were separated by TLC on Merck 60H silica gel plates and quantified by phosphorus analysis (Russell *et al.*, 1985). Phospholipids were identified by comparison of their R_f values with those of authentic standards (Sigma) in a one-dimensional solvent system containing chloroform/methanol/acetic acid/water (85:15:10:3.5). Lipids were visualized using iodine vapour (general stain), ninhydrin (for free amino groups) and molybdate reagent (for phosphate groups) as described by Kates

(1986). Quantification was established as mol % lipid phosphorus and the standard deviations of replicates were less than 5 % (Table 2).

Fatty-acid methyl esters (FAME) were prepared from lyophilized cells by acid-catalysed transmethylation (Komagata & Suzuki, 1987). FAME were analysed by gas-liquid chromatography (Sutcliffe, 1994). The percentages of each fatty acid in the profile when grown in minimal medium containing 5 % NaCl (w/v) and 4 mM phenol are given in Table 2. Analyses were performed in triplicate on three independent cultures and the standard deviations were less than 1 %.

Determination of DNA base composition

DNA was prepared from 1 ml of late exponential cultures using a QIAamp tissue kit (Qiagen). The G + C content of strain GB6^T DNA was determined to be 54 mol % by the HPLC method of Tamaoka (1994). The original characterization of [*Pseudomonas*] *doudoroffii* indicated that the G + C content of the DNA was 59.7 mol % (Baumann *et al.*, 1972), determined by the method of Mandel (1966), which appeared significantly different from that determined for strain GB6^T. However, a comparative analysis of the DNA base composition of the type strain, [*Pseudomonas*] *doudoroffii* DSM 7028^T, indicated that the G + C content of this strain was also 54 mol %. Moreover, the HPLC technique we employed was validated by using DNA prepared from an *E. coli* control.

Table 1. Differential phenotypic characteristics of isolate GB6^T, [*Pseudomonas*] *doudoroffii* DSM 7028^T and closely related bacteria

Data for *T. auensis* were taken from Fischer-Romero *et al.* (1996) and data for *A. hydrophila* were taken from Fischer-Romero *et al.* (1996) and Kaznowski (1998). L-Alanine was utilized by all four taxa. NA, Data not available.

Characteristic	[<i>Pseudomonas</i>] <i>doudoroffii</i> DSM 7028 ^T	<i>O. baumannii</i> ATCC 700832 ^T	<i>T. auensis</i> DSM 9187 ^T	<i>A. hydrophila</i> ATCC 7966 ^T
Temperature for growth (°C)	10–41	10–41	12–25	5–41
Reduction of nitrate to nitrite*	+	+	NA	+
Indole production*	—	—	—	+
Acidification of glucose*	—	—	NA	+
Arginine hydrolase present*	—	—	NA	+
Urease present*	—	—	—	NA
Gelatin hydrolysis*	—	—	—	NA
Aesculin hydrolysis*	—	—	NA	+
β -Galactosidase present*	—	—	NA	NA
Utilization of:				
Glucose*	—	—	+	+
Arabinose*	—	—	+	+
Mannose*	—	—	+	+
Mannitol*	—	—	+	+
<i>N</i> -Acetylglucosamine*	—	—	—	+
Maltose*	—	—	+	+
Gluconate*	—	—	+	+
Caprate*	+	+	NA	NA
Adipate*	—	—	NA	NA
Malate*	+	+	—	—
Citrate*	+	+	NA	—
Phenylacetate*	—	—	NA	NA
Sucrose†	—	—	+	+
Glycerol†	—	+	—	NA
Ethanol†	+	+	NA	NA
Galactose†	—	+	+	+
Betaine†	+	+	NA	NA
Sarcosine†	+	+	NA	NA
Phenol†	+	+	NA	NA
Toluene†	—	—	NA	NA
Sodium succinate†	+	+	NA	NA
L-Proline†	+	+	NA	+
L-Glutamate†	+	+	+	NA
L-Leucine†	—	—	NA	NA
L-Valine†	—	—	NA	NA
L-Tyrosine†	—	—	NA	NA
Requirement for Na ⁺	+	+‡	NA	NA
DNA G + C content (mol %)	54	54	49	58–62

* Determined by using the API20 NE kit.

† Determined in minimal medium with 4 mM substrate.

‡ Determined by Wisse & MacLeod (1989).

It is clear from the data in Tables 1 and 2 that isolate GB6^T and [*Pseudomonas*] *doudoroffii* DSM 7028^T exhibit very similar phenotypic and chemotaxonomic properties, whilst the phylogenetic analysis (Fig. 1) demonstrated that [*Pseudomonas*] *doudoroffii* can no longer be placed in the genus *Pseudomonas*, a view

suggested previously on the basis of RNA–DNA hybridization studies (De Vos *et al.*, 1989). However, in contrast with the suggestion of De Vos *et al.* (1989), our 16S rDNA analysis did not indicate that [*Pseudomonas*] *doudoroffii* belongs in the genus *Aeromonas*, and the most closely related taxon was determined to

Table 2. Comparative phospholipid compositions and fatty acid profiles of isolate GB6^T and [*Pseudomonas*] *doudoroffii* DSM 7028^T

Both organisms were grown in minimal medium containing 5% (w/v) NaCl with phenol as the sole substrate. Data for phospholipids are given as mol% phosphorus; data for fatty acids are given as percentages of total fatty acid content.

Compound	<i>O. baumannii</i> GB6 ^T	[<i>Pseudomonas</i>] <i>doudoroffii</i> DSM 7028 ^T
Phospholipids		
Diphosphatidylglycerol	3.1	Trace
Phosphatidylethanolamine	74.4	51
Phosphatidylglycerol	22.4	49
Fatty acids		
C _{12:0}	3	7
C _{16:0}	20	21.2
C _{16:1cis}	45	42
C _{18:1cis}	32	29.9

be *Tolumonas auensis*, a facultatively anaerobic Gram-negative bacterium that represents a recently described novel genus within the γ -subclass of the *Proteobacteria* (Fischer-Romero *et al.*, 1996). Intriguingly, given the taxonomic history of [*Pseudomonas*] *doudoroffii*, the description of *T. auensis* indicated that it was most closely related to *Aeromonas hydrophila* (Fischer-Romero *et al.*, 1996). Consequently, we have reanalysed the relationship between [*Pseudomonas*] *doudoroffii* DSM 7028^T and *A. hydrophila* ATCC 7966^T (GenBank accession no. X60404). The 16S rDNA similarity between the two isolates was 91.2% and did not suggest a close relationship. This conclusion is supported by phenotypic (Table 1) and chemotaxonomic (Table 2) data. The latter showed that the fatty acid profiles of the two organisms exhibited significant differences, particularly in that *Aeromonas* species possess a more complex profile including C_{13:0}, C_{14:0} and branched C_{17:0} fatty acids (Huys *et al.*, 1994), which are absent in [*Pseudomonas*] *doudoroffii*. Thus, it is clear that strains presently classified as [*Pseudomonas*] *doudoroffii* represent a novel taxon within the γ -subclass of the *Proteobacteria*, for which we propose the name *Oceanomonas* gen. nov. In addition, isolate GB6^T, on the basis of the DNA similarity, would appear to be a distinct species within this genus.

Description of *Oceanomonas* gen. nov.

Oceanomonas (O.ce.a.no.mo'nas. Gr. n. *okeanus* ocean; Gr. fem. n. *monas* monad, unit; M.L. fem. n. *Oceanomonas* ocean monad).

Cells are straight rods, 0.7–1.2 × 2.0–2.5 μ m, motile by one to four polar flagella. Gram-negative, chemo-organotrophs with an absolute requirement for Na⁺ for growth. Metabolism is obligately respiratory with molecular oxygen as the terminal electron acceptor.

The major phospholipids are phosphatidylglycerol, phosphatidylethanolamine and diphosphatidylglycerol. The G+C content is 54 mol%. The organisms are isolated from marine environments. The type species of the genus is *Oceanomonas doudoroffii* (ATCC 27123^T).

Description of *Oceanomonas doudoroffii* (Baumann *et al.* 1983) comb. nov.

Details of this taxon are given by Baumann *et al.* (1983) with the following amendments. The major fatty acids present in the cell envelope are dodecanoic, hexadecanoic, hexadecenoic and octadecenoic acids. The bacterium can grow on caprate, malate, citrate, ethanol, betaine, sarcosine, phenol, succinate, L-alanine, L-proline and L-glutamate; some strains can utilize D-ribose. The G+C content of the DNA is 54 mol%. The type strain is ATCC 27123^T (= DSM 7028^T).

Description of *Oceanomonas baumannii* sp. nov.

Oceanomonas baumannii (bau.man'ni.i. M.L. gen. n. *baumannii* after Paul and Linda Baumann, American microbiologists who first isolated these bacteria).

Aerobic Gram-negative rod (2 × 1 μ m) with up to four polar flagella. Growth occurs between 10 and 30 °C; the bacterium can also tolerate up to 7% (w/v) NaCl. Can grow on glycerol and D-galactose in addition to caprate, malate, citrate, ethanol, betaine, sarcosine, phenol, succinate, L-alanine, L-proline and L-glutamate. The DNA G+C content is 54 mol%. The major fatty acids are dodecanoic, hexadecanoic, *cis*-hexadecenoic and *cis*-octadecenoic acids. The organism was isolated from the estuary of the River Wear, Sunderland, UK. The type strain is GB6^T (= ATCC 700832^T = NCIMB 13685^T).

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