Bowmanella pacifica sp. nov., isolated from a pyrene-degrading consortium

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A taxonomic study was carried out on a strain, designated W3-3A^T, which was isolated from a pyrene-degrading consortium, enriched from sediment of the Pacific Ocean. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain W3-3A^T belonged to the genus *Bowmanella*, with the highest sequence similarity (99.0%) with *Bowmanella denitrificans* BD1^T, whereas sequence similarities with other species were less than 93%. The nucleotide sequence similarity of both *gyrB* and *rpoD* genes of strain W3-3A^T and *B. denitrificans* BD1^T was 81.1%. However, the protein sequence similarities of the *gyrB* and *rpoD* genes of strain W3-3A^T and *B. denitrificans* BD1^T were 96.1% and 91.0%, respectively. Phylogenetic trees based on these housekeeping genes showed that strain W3-3A^T and *B. denitrificans* BD1^T formed a distinct lineage in the *Gammaproteobacteria*. The DNA–DNA hybridization value between strain W3-3A^T and *B. denitrificans* BD1^T was 43%. Strain W3-3A^T could also be differentiated from *B. denitrificans* BD1^T based on the repetitive extragenic palindromic DNA-PCR fingerprint. The G+C content of the chromosomal DNA of strain W3-3A^T was 49 mol%. The combined genotypic and phenotypic data showed that strain W3-3A^T represents a novel species of the genus *Bowmanella*, for which the name *Bowmanella pacifica* sp. nov. is proposed, with the type strain W3-3A^T (=CGMCC 1.7086^T=LMG 24568^T=MCCC 1A01018^T).

During the screening of pyrene-degrading bacteria, an *Alteromonas*-like bacterium, designated strain W3-3A^T, was isolated from a pyrene-degrading consortium, enriched from sediment of the Pacific Ocean in 2002 (Wang *et al.*, 2008), and was selected for further characterization by using a polyphasic approach, including genotypic, chemo-

taxonomic and classical phenotypic characteristics. Based on these results, we consider that strain W3-3A^T should be classified as representing a novel species of the genus *Bowmanella*, which was proposed by Jean *et al.* (2006). Strains isolated in this study were stored at –80 °C in M2 medium (1^{–1} seawater: CH₃COONa, 5.0 g; tryptone, 0.5 g; yeast extract, 0.5 g; glucose, 0.5 g; sucrose, 0.5 g; sodium citrate, 0.05 g; malic acid, 0.05 g; NH₄NO₃, 1.0 g; NH₄Cl, 0.2 g; KH₂PO₄, 0.5 g; adjusted to pH 7.6) (Wang *et al.*, 2008), supplemented with 16% (v/v) glycerol for maintenance. M2 medium was used for routine cultivation of the isolates and most phenotypic tests. All cultures were incubated at 28 °C unless noted otherwise.

Genomic DNA was prepared according to the method of Ausubel *et al.* (1995) and the 16S rRNA gene was amplified by PCR using primers that have been described previously (Liu & Shao, 2005). Sequences of related taxa were obtained from the GenBank database. Phylogenetic analysis was performed using MEGA version 4 (Tamura *et al.*, 2007) after multiple alignments of data by using DNAMAN (version 5.1; Lynnon Biosoft). Distances (distance options according to the Kimura two-parameter model) and clustering with the neighbour-joining method of Saitou & Nei (1987)

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Abbreviations: *gyrB*, DNA gyrase subunit B gene; *rpoD*, DNA-directed RNA polymerase subunit D gene; rep-PCR, repetitive extragenic palindromic DNA-PCR.

The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequences reported in this study are EU440951 (*Bowmanella pacifica* W3-3A^T, 16S rRNA gene), EU441038 (*B. pacifica* W3-3A^T, partial *gyrB* gene), EU441040 (*B. pacifica* W3-3A^T, partial *rpoD* gene), EU441037 (*Bowmanella denitrificans* BD1^T, partial *gyrB* gene) and EU441039 (*B. denitrificans* BD1^T, partial *rpoD* gene).

Transmission electron micrographs of cells of strain W3-3A^T, dendrograms showing the phylogenetic positions of strain W3-3A^T and related species based on *gyrB* and *rpoD* gene sequences, and results of rep-PCR and polar lipid analysis of strain W3-3A^T and *B. denitrificans* BD1^T and a table showing the cellular fatty acid contents of strain W3-3A^T and *B. denitrificans* BD1^T are available as supplementary material with the online version of this paper.

and minimum-evolution methods of Rzhetsky & Nei (1992, 1993) were determined by using bootstrap values based on 1000 replications. As the topology of the minimum-evolution tree was similar to that obtained using the neighbour-joining method, the data generated using minimum-evolution are not shown.

A nearly full-length 16S rRNA gene sequence (1496 nt) of strain W3-3A^T was determined. As shown in Fig. 1, the phylogenetic tree based on 16S rRNA gene sequences showed that strain W3-3A^T and *Bowmanella denitrificans* BD1^T formed an independent monophyletic cluster, with a high level of similarity (99.0%); similarity values between the sequence of strain W3-3A^T and those of other related taxa were all less than 93%. The high level of 16S rRNA gene similarity confirmed that strain W3-3A^T belonged to the genus *Bowmanella*.

For further comparison of strain W3-3A^T with *B. denitrificans* BD1^T, two housekeeping genes, the DNA gyrase subunit B gene (*gyrB*) and the DNA-directed RNA polymerase subunit D gene (*rpoD*) of the two strains were sequenced using the method described by Yamamoto *et al.* (2000). The similarity values between the *gyrB* and *rpoD* gene sequences of strain W3-3A^T and *B. denitrificans* BD1^T were both 81.1%. In addition, the protein sequence similarities of the *gyrB* and *rpoD* genes of strain W3-3A^T and *B. denitrificans* BD1^T were 96.1 and 91.0%, respectively. As shown in Supplementary Figs S1 and S2 (available in IJSEM Online), phylogenetic trees based on the sequences of the two housekeeping genes showed that strain W3-3A^T and *B. denitrificans* BD1^T formed an independent monophyletic cluster, similar to that obtained based on the 16S rRNA gene sequences. Strain W3-3A^T could be differentiated from *B. denitrificans* BD1^T based on these results.

DNA–DNA hybridization experiments were performed with genomic DNA of strain W3-3A^T and *B. denitrificans* BD1^T using a method that was described previously (Liu & Shao, 2005). Genomic DNA from *Escherichia coli* DH5 α was used as an outgroup sample. Salmon sperm DNA was used as a negative control. The results showed that strain

W3-3A^T and *B. denitrificans* BD1^T had low DNA–DNA relatedness (43%), demonstrating their affiliation to different species in accordance with the cut-off value of 70% recognized by Wayne *et al.* (1987) for discrimination of bacterial species. Strain W3-3A^T and *B. denitrificans* BD1^T were compared further by using repetitive extragenic palindromic DNA-PCR fingerprint (rep-PCR). The primer BOX-A1R (5'-CTACGGCAAGGCGACGCTGACG-3') was used for rep-PCR fingerprint analysis (Versalovic *et al.* 1991). PCR was carried out with the following cycle conditions: denaturation for 5 min at 94 °C; 35 cycles of 15 s at 94 °C, 30 s at 53 °C and 8 min at 65 °C and a final extension at 65 °C for 8 min. The PCR products were separated by using agarose (2%, w/v) gel electrophoresis. The rep-PCR result is shown in Supplementary Fig. S3 (in IJSEM Online). Strain W3-3A^T showed a rep-PCR fingerprint pattern that was different from that of *B. denitrificans* BD1^T. These results further confirmed the results of the DNA–DNA hybridization.

General cell morphology was studied under an Olympus inverted microscope (Olympus IX70) using 1 day-old cultures of strain W3-3A^T grown on M2 agar medium. For electron microscopy, exponential-phase cells were harvested, suspended and absorbed on a Formvar–carbon-coated grid, and stained with phosphotungstic acid. The Gram reaction and catalase and oxidase activities were carried out according to Dong & Cai (2001). The optimal temperature for growth (using 4, 10, 20, 25, 37, 42 and 55 °C) and pH (from pH 3 to 12) were determined in M2 medium. Tolerance of NaCl was tested by using Luria–Bertani (LB) medium (10 g peptone l⁻¹ and 5 g yeast l⁻¹), supplemented with NaCl concentrations of 0, 0.5, 1, 3, 5, 7, 8, 9, 10, 12 and 15% (w/v). Other biochemical tests were carried out in duplicate using API 20NE and API ZYM strips (bioMérieux) and the Biolog GN2 MicroPlate panel, according to the manufacturers' instructions, with the adjustment that the NaCl concentration was 3.0%. With the exception of the Biolog test, *B. denitrificans* BD1^T was tested at the same time as strain W3-3A^T for comparison.

Strain W3-3A^T was found to be a Gram-negative, non-pigmented, rod-shaped bacterium that was motile by

Fig. 1. Neighbour-joining tree showing the phylogenetic positions of strain W3-3A^T and representatives of some other related taxa, based on 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 1000 replications) are shown at branch points. Bar, 0.01 nucleotide substitution rate (K_{nuc}) units.

means of at least one polar flagellum (see Supplementary Fig. S4 in IJSEM Online). The differential physiological, biochemical and chemotaxonomic characteristics between strain W3-3A^T and *B. denitrificans* BD1^T are given in Table 1.

Antibiotic susceptibility tests were performed by using disc-diffusion methods as described by Shieh *et al.* (2003). Strain W3-3A^T and *B. denitrificans* BD1^T were tested at the same time in this study. The two strains were sensitive to chloramphenicol (30 µg per disk; Oxoid), ciprofloxacin (5 µg), co-trimoxazole (25 µg), erythromycin (15 µg), rifampicin (5 µg), gentamicin (10 µg), norfloxacin (10 µg) and polymyxin B (30 IU); and resistant to ampicillin (10 µg), carbenicillin (100 µg), cefalexin (30 µg), cefazolin (30 µg), cefoperazone (30 µg), cephradine (30 µg), clindamycin (2 µg), lincomycin (2 µg), metronidazole (5 µg), minocycline (30 µg), oxacillin (1 µg), penicillin G (10 µg), ceftriaxone (30 µg), tetracycline (30 µg) and doxycycline (30 µg). The susceptibilities to other antibiotics that differentiate the two strains are shown in Table 1.

Fatty acids of whole cells of strain W3-3A^T and *B. denitrificans* BD1^T grown on 216L plate medium (1⁻¹ seawater: peptone, 10 g; yeast, 2 g; sodium acetate, 1 g; citrate, 0.5 g; agar, 15 g; pH 7.5) at 28 °C for 48 h were extracted, saponified and esterified. Analysis of the fatty acid methyl esters was performed using GC, according to the instructions of the MIDI system (Sasser, 1997). The fatty acid profile of *B. denitrificans* BD1^T was determined in parallel with strain W3-3A^T. The fatty acid profiles of the two strains are shown in Supplementary Table S1 (in

IJSEM Online). The major fatty acids of the two strains were C_{16:1}ω7c/ω6c (30.4%), C_{16:0} (23.3%), C_{17:1}ω8c (7.1%) and C_{18:1}ω7c (13.8%), accounting for >74% of the total fatty acids. The amounts of summed feature 3 (C_{16:1}ω7c/ω6c) of the two strains were different. Strain W3-3A^T has one peak corresponding to summed feature 3, which accounted for the content of 30.4%. However, *B. denitrificans* BD1^T had two close peaks that corresponded to summed feature 3, which accounted for 27.7 and 6.7%, respectively. As the fatty acid profiles of strain W3-3A^T and *B. denitrificans* BD1^T were determined under the same conditions, strain W3-3A^T could be distinguished from *B. denitrificans* BD1^T based on the differences in proportion.

Polar lipids of strain W3-3A^T and *B. denitrificans* BD1^T were extracted and separated according to the methods described by Tindall (1990). The results are shown in Supplementary Fig. S5 (in IJSEM Online). The major polar lipids of strain W3-3A^T and *B. denitrificans* BD1^T were an unidentified aminolipid, diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. Minor amounts of phosphatidylinositol and an unidentified lipid were also detected in both strains. In the study of Jean *et al.* (2006), only phosphatidylglycerol and phosphatidylethanolamine were detected.

The G+C content of the chromosomal DNA was determined according to the methods described by Mesbah & Whitman (1989), using a reversed-phase HPLC. The DNA G+C content of strain W3-3A^T was 49 mol%, similar to that of *B. denitrificans* BD1^T.

The high level of 16S rRNA gene similarity between strain W3-3A^T and *B. denitrificans* BD1^T confirmed that strain W3-3A^T belonged to the genus *Bowmanella*. However, strain W3-3A^T could be differentiated from *B. denitrificans* BD1^T based on data from the *gyrB* and *rpoD* gene sequence comparisons, DNA–DNA hybridization and rep-PCR fingerprints. On the basis of the data described above, strain W3-3A^T should be placed in a novel species of the genus *Bowmanella*, for which the name *Bowmanella pacifica* sp. nov. is proposed.

Emended description of the genus *Bowmanella* Jean *et al.* 2006

Major polar lipids are an unidentified aminolipid, diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. Minor amounts of phosphatidylinositol and an unidentified lipid are also detected.

Description of *Bowmanella pacifica* sp. nov.

Bowmanella pacifica (pa.ci'fi.ca. L. fem. adj. *pacificus* peaceful, pertaining to the Pacific Ocean).

Cells are rod-shaped, 0.6–0.8 µm wide and 1.1–1.3 µm long, and motile by means of a single polar flagellum. Positive for oxidase, catalase, gelatinase, β-glucosidase (aesculin hydrolysis) and β-galactosidase, but negative for

Table 1. Differential physiological characteristics of strain W3-3A^T (*B. pacifica* sp. nov.) and *B. denitrificans* BD1^T

Data for *B. denitrificans* BD1^T were taken from Jean *et al.* (2006). Characteristics are scored as: w, weakly positive; +, positive; –, negative.

Characteristic	Strain W3-3A ^T	Strain BD1 ^T
API 20NE*		
N-Acetyl-glucosamine, maltose	–	+
Susceptibility to antimicrobial agents*		
Furazolidone (15 µg)	+	–
Kanamycin (30 µg), neomycin (10 µg), ofloxacin (5 µg), piperacillin (100 µg), streptomycin (10 µg), vancomycin (30 µg)	–	+
Growth in 10% NaCl*	+	w
Utilization of:		
D-Galactose	–	+
Melibiose	+	–
DNA G+C content (mol%)	49	50

*The characteristics of strain BD1^T were determined in this study at the same time as strain W3-3A^T.

Gram reaction, indole production, urease and arginine dihydrolase. On M2 agar medium, produces smooth grey-white colonies with regular edges that are 2–3 mm in diameter after 72 h incubation at 28 °C, non-pigmented and slightly raised in the centre. Moderately halophilic; grows in 0–10% NaCl (optimum, 0.5–7%) and at 10–42 °C (optimum, 25–37 °C), but not at 4 or 45 °C. Growth occurs at pH 6–10 (optimum, pH 8). Capable of denitrification; does not ferment glucose. Principal fatty acids are $C_{16:1\omega7c/\omega6c}$, $C_{16:0}$, $C_{17:1\omega8c}$ and $C_{18:1\omega7c}$. Among the 95 carbon sources in the Biolog GN2 system, positive reactions were obtained for utilization of acetic acid, trehalose, gentiobiose, glycyl L-aspartic acid, inosine, lactulose, L-alanine, L-proline, sucrose, turanose, α -D-lactose, cellobiose, dextrin, D-fructose, D-mannose, melibiose, glycogen, glycyl L-glutamic acid, L-alaninamide, L-alanyl glycine, L-asparagine, L-glutamic acid, L-leucine, maltose, N-acetyl-D-glucosamine, Tween 40, Tween 80, α -cyclodextrin, α -D-glucose, β -hydroxybutyric acid and methyl β -D-glucoside. With API ZYM, positive for acid phosphatase, alkaline phosphatase, esterase lipase (C8), leucine aminopeptidase, N-acetyl- β -glucosaminidase, naphthol-AS-BI-phosphoamidase and valine aminopeptidase; weakly positive for cystine aminopeptidase, esterase (C4), trypsin and α -chymotrypsin; and negative for lipase (C14), α -fucosidase, α -galactosidase, α -glucosidase, α -mannosidase, β -galactosidase, β -glucosidase and β -glucuronidase. Sensitive to chloramphenicol (30 µg), ciprofloxacin (5 µg), co-trimoxazole (25 µg), erythromycin (15 µg), furazolidone (15 µg), gentamicin (10 µg), norfloxacin (10 µg), polymyxin B (30 IU) and rifampicin (5 µg); resistant to ampicillin (10 µg), carbenicillin (100 µg), cefalexin (30 µg), cefazolin (30 µg), cefoperazone (30 µg), cephradine (30 µg), clindamycin (2 µg), kanamycin (30 µg), lincomycin (2 µg), metronidazole (5 µg), minocycline (30 µg), neomycin (10 µg), ofloxacin (5 µg), oxacillin (1 µg), penicillin G (10 µg), piperacillin (100 µg), ceftriaxone (30 µg), streptomycin (10 µg), tetracycline (30 µg), vancomycin (30 µg) and doxycycline (30 µg). The G+C content of the DNA of the type strain is 49 mol%. Characteristics used to distinguish strain W3-3A^T from *B. denitrificans* BD1^T are given in Table 1.

The type strain, W3-3A^T (=CGMCC 1.7086^T=LMG 24568^T=MCCC 1A01018^T), was isolated from marine sediment samples collected in 2002 from the Pacific Ocean.

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