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# *Orrella amnicola* sp. nov., isolated from a freshwater river, reclassification of *Algicoccus marinus* as *Orrella marina* comb. nov., and emended description of the genus *Orrella*

Shih-Yi Sheu<sup>1</sup>, Li-Chu Chen<sup>1</sup>, Che-Chia Yang<sup>1</sup>, Aurelien Carlier<sup>2,3</sup> and Wen-Ming Chen<sup>4,\*</sup>

## Abstract

A novel Gram-negative, aerobic, non-motile, ovoid to rod-shaped bacterium, designated NBD-18<sup>T</sup>, was isolated from a freshwater river in Taiwan. Optimal growth occurred at 30 °C, at pH 6 and in the absence of NaCl. The predominant fatty acids of strain NBD-18<sup>T</sup> were C<sub>16:0</sub><sup>7</sup> summed feature 3 (C<sub>16:1</sub><sup>ω7c</sup> and/or C<sub>16:1</sub><sup>ω6c</sup>), C<sub>17:0</sub> cyclo and summed feature 8 (C<sub>18:1</sub><sup>ω7c</sup> and/or C<sub>18:1</sub><sup>ω6c</sup>). The major polar lipids were phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and phosphatidylmethylethanolamine. The major polyamine was putrescine. The major isoprenoid quinone was Q-8. The genomic DNA G+C content of strain NBD-18<sup>T</sup> was 50.9%. Strain NBD-18<sup>T</sup> was most closely related to *Orrella dioscoreae* LMG 29303<sup>T</sup> and *Algicoccus marinus* HZ20<sup>T</sup> at a 16S rRNA gene sequence similarity of 97.7%. 16S rRNA gene sequence similarity between *O. dioscoreae* LMG 29303<sup>T</sup> and *A. marinus* HZ20<sup>T</sup> was 97.7%. Phylogenetic analyses based on 16S rRNA gene sequences and an up-to-date bacterial core gene set indicated that strain NBD-18<sup>T</sup>, *O. dioscoreae* LMG 29303<sup>T</sup> and *A. marinus* HZ20<sup>T</sup> are affiliated with the same genus. Digital DNA–DNA hybridization, average nucleotide identity and average amino acid identity values among these three strains supported that they belong to the same genus and that strain NBD-18<sup>T</sup> represents a novel species. Thus, *A. marinus* HZ20<sup>T</sup> should be reclassified as *Orrella marina* comb. nov. based on the rules for priority of publication and validation. On the basis of the genotypic, chemotaxonomic and phenotypic data, strain NBD-18<sup>T</sup> represents a novel species in the genus *Orrella*, for which the name *Orrella amnicola* sp. nov. is proposed. The type strain is NBD-18<sup>T</sup> (=BCRC 81197<sup>T</sup>=LMG 31338<sup>T</sup>).

The genus *Orrella*, belonging to the family *Alcaligenaceae* within the order *Burkholderiales* of the class *Betaproteobacteria*, was proposed by Carlier *et al.* [1]. To date, this genus comprises only one recognized species: *Orrella dioscoreae*, isolated from leaf acumens of *Dioscorea sansibarensis* [1]. Members of the genus *Orrella* are characterized as Gram-negative, facultatively anaerobic, non-motile, coccobacillus-shaped and oxidase-positive. Chemotaxonomically, members of the genus *Orrella* are characterized by C<sub>16:0</sub><sup>7</sup>, C<sub>18:1</sub><sup>ω7c</sup> and summed feature 3 (C<sub>16:1</sub><sup>ω7c</sup> and/or C<sub>16:1</sub><sup>ω6c</sup>) as the predominant fatty acids, by Q-8 as the major respiratory quinone and by having a DNA G+C content of ~67.4% [1]. The genus *Algicoccus*,

belonging to the family *Alcaligenaceae* within the order *Burkholderiales* of the class *Betaproteobacteria*, was proposed by Ying *et al.* [2]. The genus *Algicoccus* presently comprises only one species with a validly published name: *Algicoccus marinus*, a marine bacterium isolated from the surface of brown seaweed *Laminaria japonica*. Cells of the genus *Algicoccus* are Gram-negative, strictly aerobic, chemo-organotrophic, non-motile, ovoid to rod-shaped, mesophilic, neutrophilic, chemotrophic, and catalase- and oxidase-positive. Chemotaxonomically, cells of the genus *Algicoccus* possess Q-8 as the sole respiratory quinone, C<sub>17:0</sub> cyclo, C<sub>16:0</sub> and summed feature 8 (C<sub>18:1</sub><sup>ω7c</sup> and/or C<sub>18:1</sub><sup>ω6c</sup>) as the predominant fatty

**Author affiliations:** <sup>1</sup>Department of Marine Biotechnology, National Kaohsiung University of Science and Technology, Kaohsiung City 811, Taiwan, ROC; <sup>2</sup>Laboratory of Microbiology, Faculty of Sciences, Ghent University, K. L. Ledeganckstraat 35, B-9000 Ghent, Belgium; <sup>3</sup>LIPM, Université de Toulouse, INRAE, CNRS, Castanet-Tolosan, France; <sup>4</sup>Laboratory of Microbiology, Department of Seafood Science, National Kaohsiung University of Science and Technology, Kaohsiung City 811, Taiwan, ROC.

\*Correspondence: Wen-Ming Chen, p62365@ms28.hinet.net

**Keywords:** *Orrella amnicola* sp. nov.; *Alcaligenaceae*; *Burkholderiales*; *Betaproteobacteria*.

**Abbreviations:** AAI, average amino acid identity; ANI, average nucleotide identity; APL, unidentified aminophospholipid; CAD, cadaverine; dDDH, digital DNA–DNA hybridization; DPG, diphosphatidylglycerol; HPUT, homoputrescine; L, unidentified lipid; ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining; PDE, phosphatidylmethylethanolamine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PUT, putrescine; Q-8, ubiquinone-8; UBCG, up-to-date bacterial core gene set.

The GenBank/EMBL/DBJ accession numbers for the 16S rRNA gene sequence and the whole genome of *Orrella amnicola* NBD-18<sup>T</sup> are MK272950 and NZ\_JAAGR000000000, respectively.

Six supplementary figures and four supplementary tables are available with the online version of this article.

**Table 1.** Differential characteristics between strains NBD-18<sup>T</sup>, *O. dioscoveae* LMG 29303<sup>T</sup> and *A. marinus* HZ20<sup>T</sup>

Strains: 1, NBD-18<sup>T</sup>; 2, *O. dioscoveae* LMG 29303<sup>T</sup>; 3, *A. marinus* HZ20<sup>T</sup>. Data were obtained in this study except the G+C content of *O. dioscoveae* LMG 29303<sup>T</sup> [1], and data for *A. marinus* HZ20<sup>T</sup> from Ying et al. [2]. +, Positive reaction; w, weakly positive reaction; -, negative reaction. The three strains are positive for oxidase, leucine arylamidase and acid phosphatase activities, and nitrate reduction. The three strains are negative for: Gram staining; motility; indol production; glucose fermentation; hydrolysis of aesculin and CM-cellulose; and arginine dihydrolase, C14 lipase, trypsin,  $\alpha$ -chymotrypsin,  $\alpha$ -galactosidase,  $\beta$ -galactosidase,  $\beta$ -glucuronidase,  $\alpha$ -glucosidase,  $\beta$ -glucosidase, *N*-acetyl- $\beta$ -glucosaminidase,  $\alpha$ -mannosidase and  $\alpha$ -fucosidase activities.

| Characteristic                              | 1                | 2                       | 3                |
|---|------------------|-------------------------|------------------|
| Isolation source                            | Freshwater river | Plant                   | Brown seaweed    |
| Colony pigmentation                         | White            | White                   | Yellow           |
| Temperature range for growth (°C) (optimum) | 25–37 (30)       | 15–40 (30)              | 4–50 (37)        |
| NaCl range for growth (%) (optimum)         | 0–0.5 (0)        | 0–0.5 (0)               | 0–10 (1–1.5)     |
| pH range for growth (optimum)               | 5.5–8 (6)        | 8–9 (8.5)               | 5–9.5 (7–7.5)    |
| Oxygen requirement                          | Strictly aerobic | Facultatively anaerobic | Strictly aerobic |
| Hydrolysis of:                              |                  |                         |                  |
| Tweens 20, 40, 60                           | –                | +                       | +                |
| Tween 80                                    | –                | –                       | +                |
| Casein                                      | +                | –                       | +                |
| Starch                                      | –                | –                       | +                |
| Urea  | –                | –                       | +                |
| Gelatin                                     | –                | –                       | +                |
| Enzymatic activities:                       |                  |                         |                  |
| Catalase                                    | –                | +                       | +                |
| Alkaline phosphatase                        | +                | –                       | +                |
| C4 esterase                                 | +                | –                       | w                |
| C8 esterase lipase                          | +                | –                       | +                |
| Valine arylamidase                          | +                | –                       | –                |
| Cystine arylamidase                         | +                | –                       | –                |
| Naphthol-AS-BI-phosphohydrolase             | +                | –                       | w                |
| Utilization of:                             |                  |                         |                  |
| Galactose                                   | +                | –                       | +                |
| Maltose                                     | +                | –                       | +                |
| Glucose                                     | –                | –                       | +                |
| Sucrose                                     | –                | –                       | +                |
| Citrate                                     | –                | +                       | +                |
| DNA G+C content (%)                         | 50.9             | 67.4                    | 55.5             |

acids, phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, an unidentified aminophospholipid and unidentified phospholipid as the major polar lipids, and a DNA G+C content of around 55.5% [2].

The 16S rRNA gene sequence similarity between *O. dioscoveae* LMG 29303<sup>T</sup> and *A. marinus* HZ20<sup>T</sup> is 97.7%. Unfortunately, there is no conclusive evidence available to elucidate the taxonomic relationship between these taxa due to the lack of experimental comparisons. In the course of a study on cultivable bacteria from a freshwater river, a bacterial strain designated NBD-18<sup>T</sup> was isolated and selected for detailed analysis. Phylogenetic analysis of the 16S rRNA gene sequence of strain NBD-18<sup>T</sup> revealed that the closest relatives of this isolate were members of the genera *Orrella* and *Algicoccus*. Strain NBD-18<sup>T</sup> occupied an intermediate position between these two genera. Sequence similarity calculations indicated that strain NBD-18<sup>T</sup> was closely related to *O. dioscoveae* LMG 29303<sup>T</sup> and *A. marinus* HZ20<sup>T</sup> at 97.7% similarity. In this study, the taxonomic relationship between these taxa was clarified by means of experimental comparisons. Based on the results of phylogenetic, phenotypic and chemotaxonomic analyses, we conclude that strain NBD-18<sup>T</sup> should be placed in the genus *Orrella* as the type strain of a novel species and that the nearest neighbour, *A. marinus* HZ20<sup>T</sup>, should be reclassified as a member of the genus *Orrella*.

## ISOLATION AND ECOLOGY

During investigations on the biodiversity of bacteria present in freshwater, a water sample (25 °C, pH 7.2, 0%, w/v, NaCl) was collected from the Dahaxi River (GPS location: 25° 02' 27" N 121° 29' 05" E) in New Taipei City, Taiwan, on 10 October 2018 (Fig. S1, available in the online version of this article). The water sample was spread on R2A agar (BD Difco) plates by the standard dilution plating method. After incubation of the plates at 25 °C for 3 days, strain NBD-18<sup>T</sup> was selected and purified as a single white colony and subjected to detailed taxonomic analyses. Strain NBD-18<sup>T</sup> was subcultured under the same conditions and stored at –80 °C in R2A broth (BD Difco) with 20% (v/v) glycerol or by lyophilization. The phylogenetically related strain *O. dioscoveae* LMG 29303<sup>T</sup> was obtained from the culture collection and used as a reference type strain and evaluated together under identical experimental conditions to those for strain NBD-18<sup>T</sup>.

## MORPHOLOGY AND PHYSIOLOGY, CULTURAL AND BIOCHEMICAL CHARACTERISTICS

Cell morphology was observed by phase-contrast microscopy (DM 2000; Leica) and transmission electron microscopy (H-7500; Hitachi) (Fig. S2) using cells grown on R2A agar at 30 °C for 2 days. The Gram Stain Set S kit (BD Difco) and the Ryu non-staining KOH method were used for testing the Gram reaction. Motility was tested by the hanging drop method [3]. The Spot Test Flagella Stain (BD Difco) was used for flagellum staining. Poly- $\beta$ -hydroxybutyrate granule accumulation was examined under light microscopy after

staining of the cells with Sudan black [4] and visualized by UV illumination after directly staining growing bacteria on plates containing Nile red [5]. Colony morphology was observed on R2A agar under a stereoscopic microscope (SMZ 800; Nikon).

The pH range for growth was determined by measuring the optical densities (absorbance at 600 nm) of R2A broth cultures. The pH of the medium was adjusted prior to sterilization to pH 4.0–9.0 (at intervals of 0.5 pH units) according to the method described by Breznak and Costilow [6]. The temperature range for growth was determined on R2A agar at 4, 10, 15, 20, 25, 30, 35, 37, 40, 45 and 50 °C. To investigate the tolerance to NaCl, R2A broth was prepared according to the formula of the BD Difco medium with NaCl concentration adjusted to 0, 0.5 and 1–6%, w/v (at intervals of 1%). Growth under anaerobic conditions was determined after incubating strain NBD-18<sup>T</sup> on R2A agar and on R2A agar supplemented with nitrate (0.1% KNO<sub>3</sub>) in anaerobic jars by using AnaeroGen anaerobic system envelopes (Oxoid) at 30 °C for 15 days. Bacterial growth was studied on R2A, nutrient, Luria-Bertani and trypticase soy agars (all from Difco) under aerobic conditions at 30 °C.

Activities of catalase, oxidase, DNase and lipase (corn oil), and hydrolysis of starch, casein, lecithin and Tweens 20, 40, 60 and 80 were determined using the methods of Tindall *et al.* [7]. Chitin hydrolysis was assessed on chitinase-detection agar [8]. Hydrolysis of carboxymethyl cellulose (CM-cellulose) was tested according to Bowman [9]. Utilization of carbon sources was investigated in a basal medium as described by Chang *et al.* [10]. Additional biochemical tests were performed using API ZYM and API 20NE kits (bioMérieux) according to the manufacturer's recommendations. Sensitivity of strain NBD-18<sup>T</sup> to antibiotics was tested by the disc diffusion method after spreading cell suspensions (0.5 McFarland) on R2A agar (BD Difco) plates at 30 °C for 2 days as described by Nokhal and Schlegel [11].

Strain NBD-18<sup>T</sup> was sensitive to nalidixic acid (30 µg), tetracycline (30 µg), novobiocin (30 µg), kanamycin (30 µg), chloramphenicol (30 µg) and streptomycin (10 µg), moderately sensitive to gentamicin (10 µg), and resistant to sulfamethoxazole/trimethoprim (23.75 and 1.25 µg, respectively), ampicillin (10 µg), rifampicin (5 µg) and penicillin G (10 U). Detailed results from phenotypic and biochemical analyses of strain NBD-18<sup>T</sup> are provided in Table 1 and in the species description.

## CHEMOTAXONOMY

The fatty acid profiles of strain NBD-18<sup>T</sup> and *O. dioscoreae* LMG 29303<sup>T</sup> were determined using cells grown on R2A agar at 30 °C for 3 days. The fatty acid methyl esters were prepared and separated according to the instructions of the Microbial Identification System (MIDI), and identified by MIDI version 6.0 and the RTSBA6.00 database [12]. The predominant cellular fatty acids (>10% of the total fatty acids) of strain NBD-18<sup>T</sup> were C<sub>16:0</sub>, summed feature 3 (C<sub>16:1</sub> ω7c and/or C<sub>16:1</sub> ω6c), C<sub>17:0</sub> cyclo and summed feature 8 (C<sub>18:1</sub> ω7c and/

**Table 2.** Cellular fatty acid composition of strains NBD-18<sup>T</sup>, *O. dioscoreae* LMG 29303<sup>T</sup> and *A. marinus* HZ20<sup>T</sup>

Strains: 1, NBD-18<sup>T</sup>; 2, *O. dioscoreae* LMG 29303<sup>T</sup>; 3, *A. marinus* HZ20<sup>T</sup>. Data for strain NBD-18<sup>T</sup> and *O. dioscoreae* LMG 29303<sup>T</sup> were obtained in this study and data for *A. marinus* HZ20<sup>T</sup> from Ying *et al.* [2]. Strain NBD-18<sup>T</sup> and *O. dioscoreae* LMG 29303<sup>T</sup> were grown on R2A agar at 30 °C for 3 days. *A. marinus* HZ20<sup>T</sup> was grown on marine agar (MA; BD) at 37 °C. Values are percentages of the total fatty acids; fatty acids that make up <1% of the total are not shown or are indicated by '-'.

| Fatty acid                  | 1    | 2    | 3    |
|-----------------------------|------|------|------|
| Straight chain              |      |      |      |
| C <sub>14:0</sub>           | 3.8  | 4.4  | -    |
| C <sub>16:0</sub>           | 31.6 | 18.2 | 27.0 |
| C <sub>18:0</sub>           | -    | 1.5  | 1.0  |
| Unsaturated                 |      |      |      |
| C <sub>17:0</sub> cyclo     | 12.0 | 10.9 | 31.5 |
| C <sub>19:0</sub> cyclo ω8c | -    | 1.2  | 1.2  |
| Hydroxy                     |      |      |      |
| C <sub>14:0</sub> 2-OH      | -    | 4.6  | -    |
| C <sub>16:1</sub> 2-OH      | 2.8  | -    | -    |
| C <sub>18:1</sub> 2-OH      | -    | 3.2  | 3.5  |
| Summed features*            |      |      |      |
| 2                           | 8.0  | 9.6  | 7.5  |
| 3                           | 26.5 | 22.4 | 2.5  |
| 5                           | -    | -    | 1.5  |
| 7                           | 1.9  | -    | -    |
| 8                           | 10.5 | 21.0 | 22.2 |

For unsaturated fatty acids, the position of the double bond is located by counting from the methyl (ω) end of the carbon chain. The *cis* isomer is indicated by the suffix *c*.

\*Summed features are groups of two or three fatty acids that cannot be separated by GLC using the MIDI system. Summed feature 2 comprises C<sub>14:0</sub> 3-OH and/or iso-C<sub>16:1</sub> 1. Summed feature 3 comprises C<sub>16:1</sub> ω7c and/or C<sub>16:1</sub> ω6c. Summed feature 5 comprises C<sub>18:0</sub> and/or C<sub>18:2</sub> ω6,9c. Summed feature 7 comprises C<sub>19:1</sub> ω7c and/or C<sub>19:1</sub> ω6c. Summed feature 8 comprises C<sub>18:1</sub> ω7c and/or C<sub>18:1</sub> ω6c.

or C<sub>18:1</sub> ω6c) (Table 2). The fatty acid profile of strain NBD-18<sup>T</sup> was similar to that of *O. dioscoreae* LMG 29303<sup>T</sup>, although there were differences in the proportions of some components. Both strains had C<sub>16:0</sub>, summed feature 3, C<sub>17:0</sub> cyclo and summed feature 8 as major fatty acids. However, the proportion of C<sub>16:0</sub> detected in strain NBD-18<sup>T</sup> was higher than that in *O. dioscoreae* LMG 29303<sup>T</sup>, and the proportion of summed feature 8 detected in strain NBD-18<sup>T</sup> was lower than that in *O. dioscoreae* LMG 29303<sup>T</sup>. In addition, comparing these data with the previously published data for *A. marinus* HZ20<sup>T</sup> [2], it is clear that the predominant fatty acids (>10%) of all three strains include C<sub>16:0</sub>, C<sub>17:0</sub> cyclo and summed feature 8. Furthermore, strain NBD-18<sup>T</sup> and *O. dioscoreae* LMG 29303<sup>T</sup>



differed from *A. marinus* HZ20<sup>T</sup> by the presence of a much higher proportion of summed feature 3.

The polar lipid profiles of strain NBD-18<sup>T</sup> and *O. dioscoreae* LMG 29303<sup>T</sup> were determined using cells grown on R2A agar at 30 °C for 2 days, and polar lipids were extracted and analysed by two-dimensional TLC according to Embley and Wait [13]. Strain NBD-18<sup>T</sup> exhibited a complex polar lipid profile consisting of phosphatidylethanolamine (PE), phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), phosphatidylmethylethanolamine (PDE), three unidentified aminophospholipids (APL1–APL3) and five unidentified lipids (L1–L5) (Fig. S3). Strain NBD-18<sup>T</sup> and *O. dioscoreae* LMG 29303<sup>T</sup> exhibited similar overall polar lipid profiles, and they had PE, PG, DPG, PDE, APL1, APL2, L1, L2 and L5 in common. However, APL3, L3 and L4 were detected in strain NBD-18<sup>T</sup> but not in *O. dioscoreae* LMG 29303<sup>T</sup>. Strain NBD-18<sup>T</sup> differed from the reference strain primarily in the presence and proportions of some minor unidentified polar lipids. In addition, when this result was compared with that of the previous analysis for *A. marinus* HZ20<sup>T</sup> [2], all three strains have PE, PG and DPG as predominant polar lipids, but *A. marinus* HZ20<sup>T</sup> has less PDE.

Polyamines were extracted from strain NBD-18<sup>T</sup> and analysis was carried out as described by Busse and Auling [14] and Busse *et al.* [15]. Cells were cultivated in R2-PYE broth (per litre: 0.75 g peptone from casein, 0.75 g yeast extract, 0.3 g K<sub>2</sub>HPO<sub>4</sub>, 0.024 g MgSO<sub>4</sub>, pH 7.2) at 30 °C for 3 days and analysed by HPLC on a D-7000 high-speed liquid chromatograph (Hitachi) and L-7420 UV-VIS detector (Hitachi). The polyamine pattern of strain NBD-18<sup>T</sup> comprised the major compound putrescine (PUT, 80.4%), a minor amount of homoputrescine (HPUT, 18.6%) and a trace amount of cadaverine (CAD, 1.0%) (Fig. S4).

The isoprenoid quinones of strain NBD-18<sup>T</sup> were extracted and purified according to the method of Collins and analysed by HPLC [16]. The respiratory quinone of strain NBD-18<sup>T</sup> was ubiquinone-8 (Q-8) (Fig. S5). The major isoprenoid quinone of *O. dioscoreae* [1] and *A. marinus* HZ20<sup>T</sup> [2] was also Q-8.

## 16S rRNA GENE PHYLOGENY

Genomic DNA was extracted using a bacterial genomic DNA purification kit (DP02-150; GeneMark), and primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1541R (5'-AAGGAGGTGATCCAGCC-3') were used for amplification of bacterial 16S rRNA genes by PCR [17, 18]. The PCR product was purified using a plus PCR clean up kit (DP04P-300; GeneMark), and then sequenced using primers 27E, 1541R, 520F and 800R [17, 18], using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems) with an ABI Prism 3730xl automated DNA analyser (Applied Biosystems). The sequenced length of the 16S rRNA gene was 1457 bp for strain NBD-18<sup>T</sup>, and this gene sequence was compared to those in EzBioCloud [19]. Multiple sequence alignments were performed with CLUSTAL W [20] (BioEdit software [21]). Phylogenetic trees were reconstructed by the

neighbour-joining (NJ) [22], maximum-likelihood (ML) [23] and maximum-parsimony (MP) [24] methods using MEGA 7 software [25]. In each case bootstrap values were calculated based on 1000 resamplings.

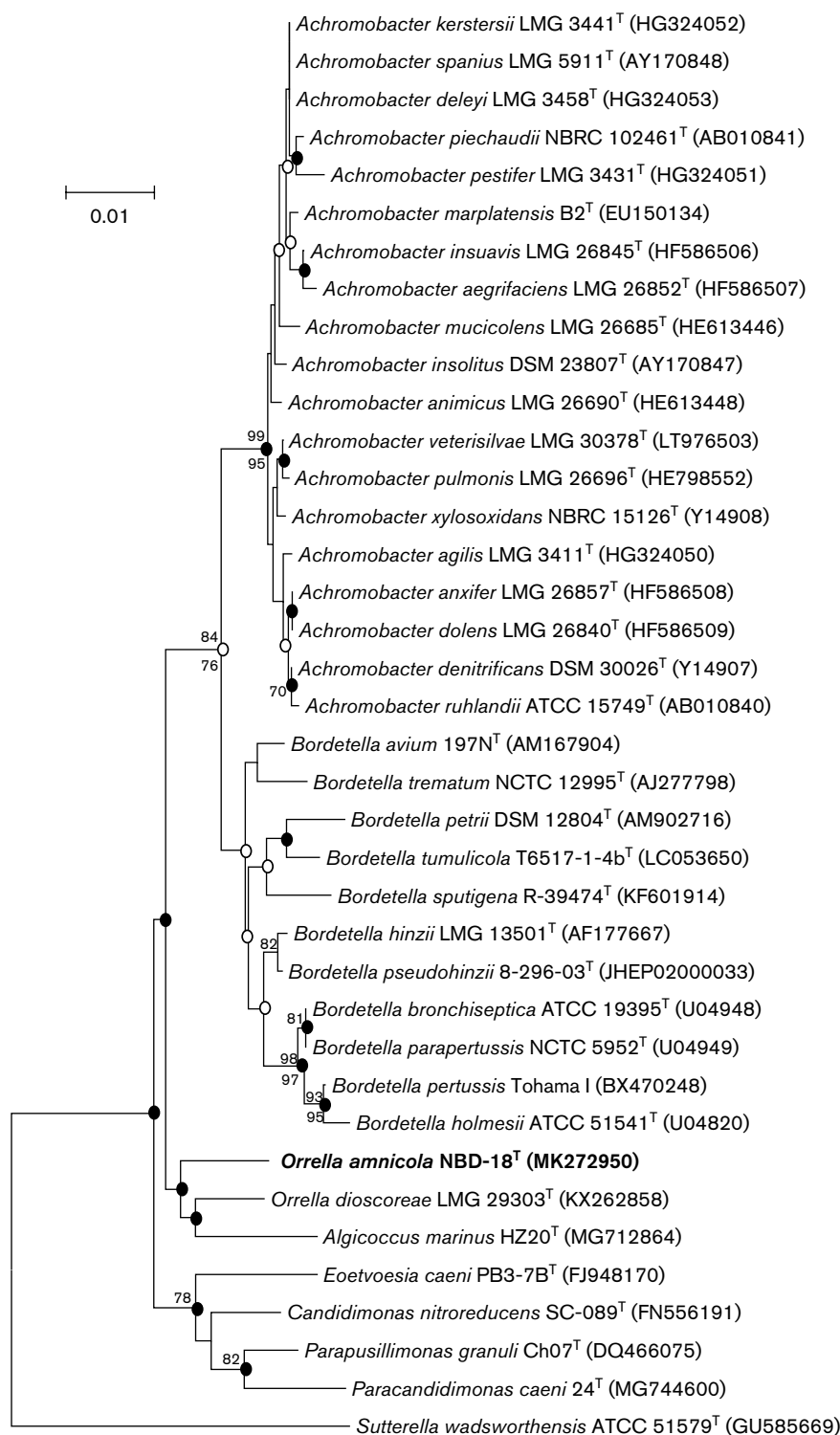
16S rRNA gene sequence analysis indicated that strain NBD-18<sup>T</sup> belongs to the family *Alcaligenaceae* of the order *Burkholderiales* in the class *Betaproteobacteria*. Sequence similarity calculations (over 1400 bp) indicated that strain NBD-18<sup>T</sup> shared high sequence similarity with *O. dioscoreae* LMG 29303<sup>T</sup> (97.7%) and *A. marinus* HZ20<sup>T</sup> (97.7%), followed by *Achromobacter* species (96.7–97.6%) and *Bordetella* species (96.3–97.3%). Sequence similarities of <97.0% were observed with the type strains of all other validly published species of the family *Alcaligenaceae*. In addition, the 16S rRNA gene sequence similarity between *O. dioscoreae* LMG 29303<sup>T</sup> and *A. marinus* HZ20<sup>T</sup> was 97.7%. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain NBD-18<sup>T</sup> formed a distinct phylogenetic branch cluster along with *O. dioscoreae* LMG 29303<sup>T</sup> and *A. marinus* HZ20<sup>T</sup>, separated from other species of the genera *Achromobacter* and *Bordetella* in the NJ tree (Fig. 1). The overall topologies of the ML and MP trees were similar.

## GENOME FEATURES

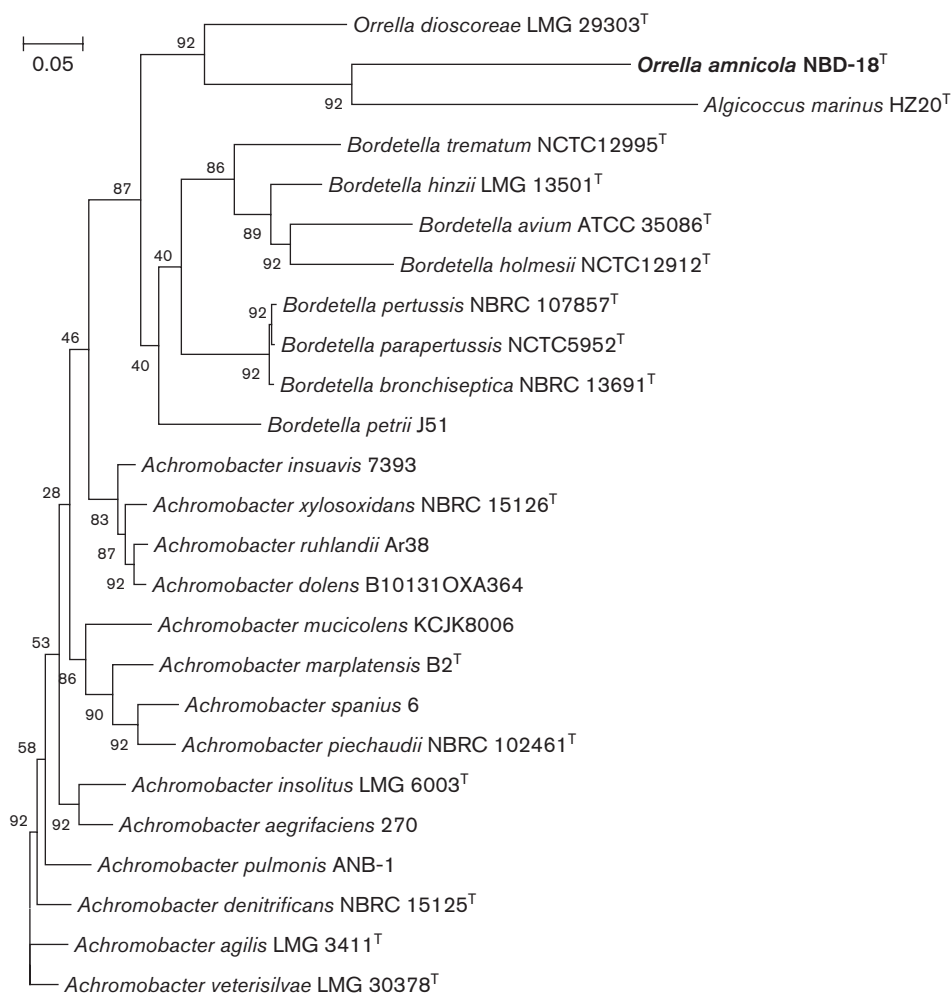
The whole genome sequence of strain NBD-18<sup>T</sup> was prepared by Genomics BioSci and Tech using the Illumina NextSeq sequencer platform and using MultiQC v1.2 for evaluating read quality [26]. The whole genome of strain NBD-18<sup>T</sup> was assembled using SPAdes (version 3.10.1) [27] and 36 contigs were obtained, with an average coverage of 2012× and an N50 size of 351635 bp. The estimated genome size was 3.18 Mb, with an average G+C content of 50.9%. Gene prediction and annotation via the Prokka pipeline [28] resulted in the identification of 2911 protein encoding genes, three rRNA genes and 42 tRNA genes.

To further explore the relationships among strain NBD-18<sup>T</sup>, *O. dioscoreae* LMG 29303<sup>T</sup> and *A. marinus* HZ20<sup>T</sup> and related genera within the family *Alcaligenaceae*, an up-to-date bacterial core gene set (UBCG) and pipeline was utilized for phylogenetic tree reconstruction [29]. The phylogenetic tree based on the coding sequences of 92 protein clusters showed that strain NBD-18<sup>T</sup> formed a distinct phylogenetic lineage with *O. dioscoreae* LMG 29303<sup>T</sup> and *A. marinus* HZ20<sup>T</sup> and distantly related to the other species of the genera *Achromobacter* and *Bordetella* in the family *Alcaligenaceae* (Fig. 2), which supported that strain NBD-18<sup>T</sup>, *O. dioscoreae* LMG 29303<sup>T</sup> and *A. marinus* HZ20<sup>T</sup> should be assigned to the same genus.

Following the proposed minimal standards for the use of genome data for the taxonomy of prokaryotes [30], both the digital DNA–DNA hybridization (dDDH) and the average nucleotide identity (ANI) between strain NBD-18<sup>T</sup> and *O. dioscoreae* LMG 29303<sup>T</sup> and *A. marinus* HZ20<sup>T</sup> were determined (Table S1). The estimated genome-sequence-based dDDH values were calculated as described by Meier-Kolthoff *et al.* [31]. The dDDH values between strain NBD-18<sup>T</sup> and



**Fig. 1.** NJ phylogenetic tree based on 16S rRNA gene sequences showing the position of *O. amnicola* NBD-18<sup>T</sup> and related taxa in the family *Alcaligenaceae*. Numbers at nodes are bootstrap percentages  $\geq 70\%$  based on the NJ (above nodes) and MP (below nodes) tree-making algorithms. Filled circles indicate branches of the tree that were also recovered using the ML and MP tree-making algorithms. Open circles indicate that the corresponding nodes were also recovered in the tree generated with the MP algorithm. *Sutterella wadsworthensis* ATCC 51579<sup>T</sup> was used as an out-group. Bar, 0.01 substitutions per nucleotide position.



**Fig. 2.** Phylogenetic tree inferred using UBCGs (concatenated alignment of 92 core genes) showing the position of *O. amnicola* NBD-18<sup>T</sup> and closely related taxa within the family *Alcaligenaceae*. The number of single gene trees supporting a branch in a UBCG tree is calculated and designated the Gene Support Index (GSI). The GSIs are given at branching points. Bar, 0.05 substitutions per position.

*O. dioscoreae* LMG 29303<sup>T</sup> and *A. marinus* HZ20<sup>T</sup> were 21.0 and 19.4%, respectively, which are below the threshold of 70% for species delineation [32]. ANI calculations were performed by OrthoANI analysis [33], which gave OrthoANI values of 69.4 and 68.3% compared to *O. dioscoreae* LMG 29303<sup>T</sup> and *A. marinus* HZ20<sup>T</sup>, which were lower than the 95–96% cut-off values previously proposed for species delimitation [34]. In addition, average amino acid identity (AAI) calculations were performed (<http://enve-omics.ce.gatech.edu/>), which gave AAI values of 69.6 and 68.0% compared to *O. dioscoreae* LMG 29303<sup>T</sup> and *A. marinus* HZ20<sup>T</sup>. The AAI value between *O. dioscoreae* LMG 29303<sup>T</sup> and *A. marinus* HZ20<sup>T</sup> was 66.3%. All values were below the threshold of 90% for species boundaries and above the threshold of 60% for genus boundaries [35]. Based on these data, strain NBD-18<sup>T</sup>, *O. dioscoreae* LMG 29303<sup>T</sup> and *A. marinus* HZ20<sup>T</sup> should be placed within the same genus and strain NBD-18<sup>T</sup> represents a novel species.

For further comparative analyses, the genome sequences of strain NBD-18<sup>T</sup>, *O. dioscoreae* LMG 29303<sup>T</sup> and *A. marinus*

HZ20<sup>T</sup> were annotated by Rapid Annotation of microbial genomes using Subsystem Technology (RAST) [36, 37]. An overview of the genome characteristics of these strains is given in Table S2. Variations in gene content between strain NBD-18<sup>T</sup>, *O. dioscoreae* LMG 29303<sup>T</sup> and *A. marinus* HZ20<sup>T</sup> may give some clues about adaptations to distinct environments (Table S3). The three strains possessed genes putatively encoding proteins associated with biotin, thiamine and pyridoxine (vitamin B6) biosynthesis. However, only *O. dioscoreae* LMG 29303<sup>T</sup> had genes putatively encoding proteins related to flavodoxin, and only strain NBD-18<sup>T</sup> had no genes putatively encoding proteins related to pterin carbinolamine dehydratase. The three strains harboured genes putatively encoding proteins associated with multidrug resistance efflux pumps, cobalt–zinc–cadmium resistance and beta-lactamase for resistance to antibiotics and toxic compounds, but only *O. dioscoreae* LMG 29303<sup>T</sup> had genes involved in tripartite systems of multidrug resistance. Concerning the membrane transport systems, the three strains showed some shared

genes in connection with the type II (widespread colonization island) transport system, but only *O. dioscoreae* LMG 29303<sup>T</sup> had genes associated with the type II (general secretion pathway), type IV (conjugative transfer), type V (two partner secretion pathway), type VI secretion system and type VII (chaperone/usher pathway) transport systems.

In addition, only *O. dioscoreae* LMG 29303<sup>T</sup> and *A. marinus* HZ20<sup>T</sup> possessed genes putatively encoding proteins associated with pterin carbinolamine dehydratase, multi-subunit cation transporter, toxin–antitoxin replicon stabilization system, ectoine biosynthesis and regulation, glutathione-dependent pathway of formaldehyde detoxification, hemin transport system and *N*-linked glycosylation. These related genes present only in *O. dioscoreae* LMG 29303<sup>T</sup> and *A. marinus* HZ20<sup>T</sup> but not in strain NBD-18<sup>T</sup> are listed in Table S4. In contrast, strain NBD-18<sup>T</sup> possessed several genes putatively encoding an enzyme related to bacterial UmuCD system [RecA protein, SOS-response repressor and protease LexA (EC 3.4.21.88), error-prone, lesion bypass DNA polymerase V (UmuC) and repair protein UmuD] and RecBCD pathway [exodeoxyribonuclease V alpha, beta and gamma chains (EC 3.1.11.5)] for DNA repair, several genes putatively encoding proteins associated with formate dehydrogenase [formate dehydrogenase O alpha, beta and gamma subunits (EC 1.2.1.2)] and carbon monoxide oxidation [carbon monoxide dehydrogenase small, medium and large chains (EC 1.2.99.2), xanthine and CO dehydrogenase maturation factor (XdhC/CoxF family), CTP:molybdopterin cytidyltransferase] for respiration. However, *O. dioscoreae* LMG 29303<sup>T</sup> and *A. marinus* HZ20<sup>T</sup> did not have these genes.

Regarding metabolism of aromatic compounds, amino acid and derivatives and various carbohydrates, the three strains showed different patterns. The most obvious differences are that only *O. dioscoreae* LMG 29303<sup>T</sup> and *A. marinus* HZ20<sup>T</sup> had genes putatively encoding proteins associated with salicylate ester degradation, arginine deiminase pathway, leucine degradation, HMG-CoA synthesis and HMG-CoA metabolism (Table S4), and only *O. dioscoreae* LMG 29303<sup>T</sup> had genes putatively encoding enzymes associated with trehalose, maltose, maltodextrin, malonate, acetoin, butanediol, glycogen, D-galactonate, D-galactarate, D-glucarate, D-glycerate, D-gluconate and ketogluconate metabolism. Furthermore, only strain NBD-18<sup>T</sup> possessed genes putatively encoding enzymes related to the Entner–Doudoroff pathway [pyruvate kinase (EC 2.7.1.40), enolase (EC 4.2.1.11), phosphoglycerate kinase (EC 2.7.2.3), phosphoglycerate mutase (EC 5.4.2.1), NAD-dependent glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12), 2-dehydro-3-deoxygluconate kinase (EC 2.7.1.45), 4-hydroxy-2-oxoglutarate aldolase (EC 4.1.3.16) and 2-dehydro-3-deoxyphosphogluconate aldolase (EC 4.1.2.14)] for central carbohydrate metabolism (Table S3).

Recently, through the complete genome sequence of *A. marinus* HZ20<sup>T</sup>, it was found that although *A. marinus* HZ20<sup>T</sup> exists in the brown seaweed-abundant environment, it does not have algal-polysaccharide-degrading abilities

[38]. In this study, the genome sequences of strain NBD-18<sup>T</sup>, *O. dioscoreae* LMG 29303<sup>T</sup> and *A. marinus* HZ20<sup>T</sup> were annotated by RAST, which confirmed that these three strains have very few enzymes related to polysaccharide metabolism. At the same time, it is also consistent with the fact that all three strains have genes related to polyhydroxybutyrate metabolism, suggesting that they can synthesize or utilize polyhydroxybutyrate to regulate carbon sources.

Additionally, the percentages of genes of strain NBD-18<sup>T</sup> shared with *O. dioscoreae* LMG 29303<sup>T</sup> and *A. marinus* HZ20<sup>T</sup> were estimated. On the basis of the data obtained from EDGAR 2.0 [39], an enhanced software platform for comparative gene content analyses, strain NBD-18<sup>T</sup>, *O. dioscoreae* LMG 29303<sup>T</sup> and *A. marinus* HZ20<sup>T</sup> had 1283 genes in common, which is about 43.7% of the total number of genes in strain NBD-18<sup>T</sup>. Strain NBD-18<sup>T</sup> shared 1545 genes (52.7%) with *O. dioscoreae* LMG 29303<sup>T</sup> and 1657 genes (56.5%) with *A. marinus* HZ20<sup>T</sup>. However, 1015 genes were species-specific genes for strain NBD-18<sup>T</sup>, accounting for about 34.6% of the total number of genes in strain NBD-18<sup>T</sup> (Fig. S6). Because strain NBD-18<sup>T</sup>, *O. dioscoreae* LMG 29303<sup>T</sup> and *A. marinus* HZ20<sup>T</sup> were isolated from freshwater river, plant and brown seaweed, respectively, different metabolic abilities are probably crucial for adaptation in their respective environments.

## TAXONOMIC CONCLUSION

Phenotypic examination revealed many common traits between the novel strain and the reference strains. However, strain NBD-18<sup>T</sup> could be clearly differentiated from its two closest relatives by its inability to hydrolyse Tweens 20, 40 and 60, by the absence of catalase activity, by the presence of valine arylamidase and cystine arylamidase activities, and by the inability to utilize citrate as a carbon source (Table 1). Some features of strain NBD-18<sup>T</sup> such as the lower pH range (pH 6) for optimal growth, the inability to grow at lower temperature (<25 °C), the inability to grow under anaerobic conditions, the ability to hydrolyse casein, the presence of alkaline phosphatase, C4 esterase, C8 esterase lipase and naphthol-AS-BI-phosphohydrolase activities, and the ability to utilize galactose and maltose as carbon sources, distinguish this novel strain from *O. dioscoreae* LMG 29303<sup>T</sup>. In addition, several phenotypic properties, such as its colony pigmentation, its lower optimal growth temperature (30 °C), NaCl concentration (0%) and pH (pH 6), the inability to grow at higher temperature (>37 °C), at higher NaCl concentrations (>0.5%) and at higher pH (>pH 8), the inability to hydrolyse Tween 80, starch, urea and gelatin, and the inability to utilize glucose and sucrose as carbon sources, may be helpful in separating strain NBD-18<sup>T</sup> from *A. marinus* HZ20<sup>T</sup> (Table 1).

On the basis of the data obtained from 16S rRNA gene sequence and whole genome sequence comparisons, strain NBD-18<sup>T</sup> occupies a distinct position within the genus *Orrella*. This phylogenetic insight is supported by the unique combination of chemotaxonomic and biochemical characteristics of the novel strain. It is clear from the phylogenetic and phenotypic data that strain NBD-18<sup>T</sup> represents a novel



species of the genus *Orrella*. The name *Orrella amnicola* sp. nov. is proposed for this taxon. *Orrella* was proposed by Carlier et al. in 2017 [1], and *Orrella* and *O. dioscoreae* were validated in January 2019 [40]. However, *A. marinus*, a marine bacterium isolated from the surface of brown seaweed, was described by Ying et al. in 2019 [2], and *Algicoccus* and *A. marinus* were validated in November 2019 [40]. Based on the rules for priority of publication and validation, *A. marinus* HZ20<sup>T</sup> should be reclassified as *Orrella marina* comb. nov. Furthermore, it is necessary to amend the description of the genus *Orrella* given by Carlier et al. [1] with the reclassification of *A. marinus* HZ20<sup>T</sup> and data for the description of *O. dioscoreae* LMG 29303<sup>T</sup> in this study.

## EMENDED DESCRIPTION OF THE GENUS *ORRELLA* CARLIER ET AL. 2019

*Orrella* (Orr.e'lla. N.L. fem. n. *Orrellla* from M. Young Orr, the botanist who first described the leaf glands of *Dioscorea sansibarensis*).

Cells are Gram-negative, strictly aerobic or facultatively anaerobic, non-motile, ovoid to rod-shaped, chemo-organotrophic and oxidase-positive. Growth occurs at 4–50 °C, at pH 5–9.5 and with 0–10% (w/v) NaCl. Nitrate can be reduced to nitrite. The dominant fatty acids (>10%) are C<sub>16:0</sub>, C<sub>17:0</sub> cyclo, summed feature 8 (C<sub>18:1</sub> ω7c and/or C<sub>18:1</sub> ω6c) and/or summed feature 3 (C<sub>16:1</sub> ω7c and/or C<sub>16:1</sub> ω6c). The major polar lipids are phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and/or phosphatidyl dimethylethanolamine. The main respiratory quinone is Q-8. The DNA G+C content is 50.9–67.4%. 16S rRNA gene sequence analysis indicates that the genus *Orrella* is a member of the family *Alcaligenaceae*. The type species is *Orrella dioscoreae*.

## DESCRIPTION OF *ORRELLA MARINA* COMB. NOV

*Orrella marina* (ma.ri'na. L. fem. adj. *marina* of or belonging to the sea, marine).

Basonym: *Algicoccus marinus* Ying et al. 2019.

The description is that given for *Algicoccus marinus* by Ying et al. [2].

The type strain, HZ20<sup>T</sup> (=MCCC 1K3465<sup>T</sup>=KCTC 62330<sup>T</sup>), was isolated from the surface of brown seaweed *Laminaria japonica* in the East China Sea.

## DESCRIPTION OF *ORRELLA AMNICOLA* SP. NOV.

*Orrella amnicola* [am.ni'co.la. L. masc. n. *amnis* a stream, a small river; L. suff. *-cola* (from L. masc. or fem. n. *incola*) a dweller, an inhabitant; N.L. fem. n. *amnicola* an inhabitant of a river].

Cells are Gram-negative, aerobic, non-motile, ovoid to rod-shaped and chemo-heterotrophic. Cells grow well on R2A

agar, but not on Luria-Bertani agar, nutrient agar or trypticase soy agar. Cells do not grow under anaerobic conditions. After 48 h of incubation on R2A agar at 30 °C, mean cell width is 0.5–0.9 μm and mean cell length is 1.4–1.8 μm. Colonies are white, convex, round and smooth with entire edges. Colonies are 1.1–2.0 mm in diameter on R2A agar after 48 h of incubation at 30 °C. Growth occurs at 25–37 °C (optimum, 30 °C), at pH 5.5–8 (optimum, pH 6) and with 0–1% (w/v) NaCl (optimum, 0%). Positive for poly-β-hydroxybutyrate accumulation. Positive for oxidase activity and negative for catalase activity. Positive for hydrolysis of casein, chitin and DNA. Negative for hydrolysis of starch, CM-cellulose, lecithin, corn oil and Tweens 20, 40, 60 and 80. In API 20NE tests, positive for nitrate reduction, and negative for indole production, glucose fermentation, arginine dihydrolase and β-galactosidase (PNPG) activities, and hydrolysis of urea, aesculin and gelatin. In API ZYM tests, positive for alkaline phosphatase, C4 esterase, C8 esterase lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase activities but negative for C14 lipase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase (ONPG), β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities. Growth under aerobic conditions is positive on: maltose, D-galactose, N-acetyl-D-glucosamine, Tween 40, Tween 60, glycerol, gluconate, caprate, adipate, malate, L-leucine and L-asparagine, but not on: dextrin, D-glucose, cellobiose, D-fructose, sucrose, D-mannose, D-rhamnose, trehalose, L-arabinose, raffinose, Tween 20, Tween 80, D-sorbitol, D-mannitol, adonitol, acetate, citrate, L-alanine, L-phenylalanine, L-ornithine, L-aspartic acid, L-glutamic acid, L-serine, L-threonine, L-proline and L-histidine. The major fatty acids are C<sub>16:0</sub>, summed feature 3 (C<sub>16:1</sub> ω7c and/or C<sub>16:1</sub> ω6c), C<sub>17:0</sub> cyclo and summed feature 8 (C<sub>18:1</sub> ω7c and/or C<sub>18:1</sub> ω6c). The polar lipid profile consists of phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidyl dimethylethanolamine, three unidentified aminophospholipids and five unidentified lipids. The polyamine pattern contains the major compound putrescine, minor amounts of homoputrescine and traces of cadaverine. The major isoprenoid quinone is Q-8.

The type strain, NBD-18<sup>T</sup> (=BCRC 81197<sup>T</sup>=LMG 31338<sup>T</sup>), was isolated from the Dahanxi River in New Taipei City, Taiwan. The DNA G+C content of the type strain is 50.9%. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence and the whole genome of NBD-18<sup>T</sup> are MK272950 and NZ\_JAAGRN000000000, respectively.

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### Conflicts of interest

The authors declare that there are no conflicts of interest.

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