**Notoacmeibacter marinus** gen. nov., sp. nov., isolated from the gut of a limpet and proposal of *Notoacmeibacteraceae* fam. nov. in the order *Rhizobiales* of the class *Alphaproteobacteria*

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**Abstract**

A Gram-stain-negative, short rod-shaped and non-flagellated bacterium, named strain XMTR2A4T, was isolated from the gut of a marine limpet, *Notoacmea schrenckii* on intertidal rocks. Colonies were small, light grey and circular. Catalase- and oxidase-positive. Growth was observed at 15 to 37 °C (optimum 28–30 °C), with salinity range from 0.5 to 9 % (optimum 1–2 %), and at pH 6 to 9 (optimum pH 7). The almost full-length 16S rRNA gene of strain XMTR2A4T had the highest sequence similarity of 93.7 % with *Mycoplasma ramosa* DSM 7292T, and of 93.5, 93.5 and 93.4 % with *Chelatobacter intermedius* CC-MHSW-5T, *Ensifer fredii* ATCC 35423T and *Phyllobacterium myrsinacearum* IAM 13584T, respectively. Phylogenetic analysis showed that strain XMTR2A4T formed a tight cluster with a cultured but uncharacterized strain, YP382-1-A, which was deeply separated from the species within the order *Rhizobiales* in the class *Alphaproteobacteria*. The predominant fatty acid of strain XMTR2A4T was summed feature 8 (C\(\_\_\)18:0 7c and/or C\(\_\_\)18:0 6c; 83.4 %). Ubiquinone-10 (Q-10) was detected as the sole respiratory quinone. The polar lipids were identified as phosphatidylethanolamine, phosphatidylglycerol, phosphatidyldicholine, diphasphatidylglycerol, phosphatidylmonomethylethanolamine, an unidentified phospholipid and three unknown lipids. The genome size was about 3.6 Mbp and the G+C content was 61.5 mol%. Combining the results above, it was ascertained that strain XMTR2A4T represents a novel species of a new genus in the order *Rhizobiales*, for which the name *Notoacmeibacter marinus* gen. nov., sp. nov. is proposed. The type strain of the type species is XMTR2A4T (=MCCC 1A01882K=KCTC 52427T). A novel family in the order *Rhizobiales*, named *Notoacmeibacteraceae* fam. nov., is also proposed to accommodate the new genus.

The *Alphaproteobacteria* is a class of highly diverse bacteria in the phylum *Proteobacteria*, with important biological functions. This class, to be widely recognized, contains 12 orders appearing in 31 families, which are comprehensively characterized according to the EzTaxon database [1] and LPSN resources (www.bacterio.net/). Among the orders in this class, *Rhizobiales* was the most diverse, encompassing 14 families.

During our investigation of the gut bacteria of marine limpets on intertidal rocks, isolate XMTR2A4T was obtained and represented a novel taxa affiliated with the order *Rhizobiales* in the class *Alphaproteobacteria*. In this study, we determined the taxonomic status of strain XMTR2A4T using a polyphasic approach.

The limpet, *Notoacmea schrenckii* was collected in March 2016 from intertidal rocks located at the coast of Xiamen, Fujian province, China. The whole visceral mass was cut after immersing in 70 % ethanol to disinfect the surface of whole body, homogenized in sterile seawater, and swabbed onto MS agar medium composed of marine broth 2216 (BD) and agar (BD), which were autoclaved separately and mixed when cooled below 50 °C [2]. The agar plate was incubated at 25 °C for 5 days. The isolate, XMTR2A4T, was picked and streaked twice in order to obtain a pure culture, and stored with 20 % glycerol (v/v) at −80 °C.

Genomic DNA of strain XMTR2A4T was extracted using the Bacterial Genomic Extraction Kit (SBS) following the manufacturer’s instructions. The 16S rRNA gene was...
amplified using the bacterial universal primers, Eubac27F and 1492R using Ex Taq in a 50 µl amplification system (TaKaRa). The resulting PCR product was purified and ligated into the PMD18-T vector (TaKaRa), and transferred into chemically competent *Escherichia coli* DH5α cells. Positive clones were screened and the insert fragment was determined using the vector universal primer RV-M and dideoxynucleotide sequencing. About 1 gigabase (G) of paired-end reads (2 × 150 bp) were retrieved and assembled using SPAdes v.3.7.0 with the default settings [4]. The contigs with sequence lengths longer than 1000 bp were kept. The genome size was about 3.6 Mbp.

The draft genome of strain XMTR2A4^T* was identified using the EzTaxon database [1] and the BLAST against the nucleotide collection (nr/nt) database (NCBI, www.ncbi.nlm.nih.gov/) at the same time. The search results showed that strain XMTR2A4^T had the highest 16S rRNA gene sequence identity (99%) with a cultured but uncharacterized bacterium *Mycoplana ramosa* strain YP382-1-A (GenBank accession number KU764383), isolated from marine sediment of South Korea, and other strains showing ≤94% identities. EzTaxon searching analysis showed that strain XMTR2A4^T* had the highest 16S rRNA gene sequence similarity of 93.7% with *Mycoplana ramosa* DSM 7292^T*, and 93.5, 93.5 and 93.4% with *Chelatovorans intermedius* CC-MHSW-5^T*, *Ensifer fredii* ATCC 35423^T* and *Phyllobacterium myrsinacearum* IAM 13584^T*, respectively.

Phylogenetic analysis was carried out by retrieving the 16S rRNA gene sequences with high similarities in the order Rhizobiales. Two different phylogenetic trees were reconstructed using MEGA 6.0 software package [3]: maximum-likelihood and neighbour-joining. The node support of the tree topology was evaluated using bootstrapping estimation of 1000 replicates for each method. The best substitution model (GTR+G+I) for maximum-likelihood was determined under the lowest BIC selection scores (Bayesian Information Criterion) and the maximum lnl value. Phylogenetic analysis indicated that strain XMTR2A4^T* formed a tight clade with a cultured but uncharacterized bacterium *Mycoplana ramosa* strain YP382-1-A, and was deeply separated from the species within the order Rhizobiales, suggesting that the two strains may represent a novel genus of a novel family (Figs 1 and S1, available in the online Supplementary Material).

The draft genome of strain XMTR2A4^T* was determined using the Illumina Hiseq 2500 platform (Shanghai Majorbio BioPharm Technology) according to the manufacturer’s instructions. About 1 gigabase (G) of paired-end reads (2 × 150 bp) were retrieved and assembled using SPAdes v.3.7.0 with the default settings [4]. The contigs with sequence lengths longer than 1000 bp were kept. The genome size was about 3.6 Mbp. The genome DNA G+C content was determined to be 61.5 mol% calculated using QUAST [5]. This G+C content was within the range of examined strains in the order Rhizobiales (Table 1). The complete sequence of the 16S rRNA gene was annotated from the assembled genome via RNAmmer 1.2 Server (www.cbs.dtu.dk/services/RNAmer/). The complete sequence (1479 bp) fully and exactly covered the sequence obtained via PCR clone sequencing (1413 bp). The coding genes region of strain XMTR2A4^T* was identified using Prodigal [6]. A whole-genome-based phylogenetic tree was reconstructed based on the protein sequences using CVTree 3 [7]. This tree showed that strain XMTR2A4^T* formed an independent monophyletic clade paralleled with the species in the families *Rhizobiaceae*, *Brucellaceae* and *Phyllobacteriaceae* within the order *Rhizobiales* of *Alphaproteobacteria* (Fig. S2), supporting that the strain represented a family-level taxa in agreement with the result of the 16S rRNA gene phylogeny.

Colonies of strain XMTR2A4^T* cultured on MS agar medium at 28 °C for 3 days were small (0.5 mm in diameter), circular and light grey (Fig. S3). The morphology of bacterial cells was observed using transmission electron microscopy (JEM-2100; JEOL) after negatively staining. Strain XMTR2A4^T* was a short-rod bacterium, 2.0 × 0.7 µm, and lacked flagellum (Fig. S4). Oxidase was tested using the oxidase reagent (bioMérieux) to observe the simultaneous colour change of the colony stabilized on a filter member. Catalase activity was detected by using the observation of bubble generation in 10% H₂O₂ solution. The results showed that activities of oxidase and catalase were both positive. The growth conditions were tested as follows. The growth temperature was set to 4, 10, 15, 20, 25, 30, 34, 37, 40, 45, 50 and 55 °C. The strain was maintained in marine broth 2216 for a week. The optimal salinity was determined using the basic MB medium formula without NaCl, supplemented with NaCl at 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 18 and 20% (w/v). The optimal pH was tested in MB medium ranging from pH 3 to 11 at intervals of one pH unit, adjusted with citrate/phosphate buffer (for pH 3–7), Tris/HCl buffer (pH 8–9) or sodium carbonate/sodium bicarbonate buffer (pH, 10–11). In order to identify whether the strain could produce poly-β-hydroxybutyrate (PHB) inside the bacterial cell, Nile blue A staining was applied according to a previous study [8], and was observed using fluorescence microscopy (DM6000 B; Leica Microsystems). The result confirmed that strain XMTR2A4^T* contains PHB as the bacterial cells did produce fluorescence at the excitation wavelength of 460 nm.

To analyse the physiological and biochemical characteristics and fatty acids profiles, type strains of six closely related species, *Mycoplana ramosa* DSM 7292^T* (=CGMCC 4.1474^T*) and *Mycoplana dimorph a NBRC 13291^T* (=CGMCC 4.1473^T*) [9], *Ensifer fredii* USDA 205^T* (=CGMCC 1.2545^T*) [10], *Phyllobacterium myrsinacearum* LMG 2t2^T* (=MCCC 1A03269^T*) [11], *Hoeflea marina* LMG 128^T* (=MCCC 1A06445^T*) [12], and *Nitratireductor indicus* CC115^T* (=MCCC 1A01260^T*) [13] were used as reference strains, and were obtained from China General Microbiological Culture Collection Center (CGMCC) or Marine Culture Collection of China (MCCC). To obtain enough cell biomass, strain XMTR2A4^T* and the reference strains were firstly cultivated on MS agar plates at 28 °C for 3 days except *P. myrsinacearum* LMG 2t2^T* which was
cultured for 5 days since it grows relatively slowly. Then cells were collected and suspended with sterile seawater, and inoculated into API 20NE, API 20E and API ZYM test strips according to the manufacturer’s instructions. Test strips were incubated at 28 °C for determining the physiological properties. The detailed phenotypic properties are summarized in Table 1. To measure the fatty acids of cells, strain XMTR2A4^T and the reference strains were cultured in MB at 28 °C for 3 days. Bacterial biomasses were obtained by centrifugation at 6000 r.p.m. for 10 min. The cellular fatty acids were extracted from the cells and identified following the standard MIDI protocol (Sherlock Microbial Identification System, version 6B). The predominant fatty acids of strain XMTR2A4^T consisted of summed feature 8 (C_{18:1}ω7c and/or C_{18:1}ω6c, 83.4%), which was present in a higher amount than in the members of other closely related families. The detailed fatty acid compositions of strain XMTR2A4^T and the reference strains are listed in Table 2.

For the isoprenoid quinone and polar lipids analyses, strain XMTR2A4^T was cultured in MB at 25 °C and the cells were collected by centrifugation. The isoprenoid quinone of this strain was measured using reversed-phase HPLC as previously described [14]. The ubiquinone system was detected as the sole ubiquinone Q-10, in agreement with the ubiquinone system previously described [14]. The ubiquinone system was detected as the sole ubiquinone Q-10, in agreement with the ubiquinone system previously described [14].

![Fig. 1. Neighbour-joining phylogenetic tree reconstructed based on the 16S rRNA gene sequences and indicating the relationship of strain XMTR2A4^T with the type strains in the order Rhizobiales. Bootstrap analysis was carried out 1000 replicates; branch node values <50 are not shown.](http://www.microbiologyresearch.org)
thin-layer plates were used in TLC analysis. The TLC plates were sprayed with sulfuric acid/ethanol (1 : 2, v/v) followed by heating at 150 °C for 2 min to detect phospholipids and glycolipids (Fig. S5a). The TLC plates were also visualized by treating the plates with 10% (w/v) molybdatophosphoric acid followed by heating at 150 °C for 5 min (Fig. S5b). The predominant polar lipids of strain XMTR2A4T were identified as phosphatidylethanolamine, phosphatidylcholine, phosphatidylglycerol, diphasphatidylglycerol and phosphatidylmonomethylethanolamine, which were frequently detected in the species within the families Phyllobacteriaceae, Brucellaceae and Rhizobiacae (Fig. S5 and Table S1).

Combining the above results, especially the genomic features, strain XMTR2A4T represents a novel species of a new genus within a novel family of the order Rhizobiales in the class Alphaproteobacteria. Therefore, the name Notoacmeibacter marinus gen. nov., sp. nov. is proposed, belonging to a novel family with the proposed name Notoacmeibacteriaceae fam. nov. in the order Rhizobiales.

### DESCRIPTION OF NOTOACMEIBACTER GEN. NOV.

Notoacmeibacter (No.to.ac.me.i.bac’ter. N.L. fem. n. Notoacmea a limpet genus; N.L. masc. n. bacter a rod; N.L. masc. n. Notoacmeibacter a rod from Notoacmea).

Gram-stain-negative, oxidase- and catalase-positive. Nitrate can be reduced to nitrite. Contains PHB inside the cells. Ubi-
quione-10 (Q-10) is the sole isoprenoid quinone. The major fatty acid is summed feature 8 (C18:1ω7c and/or C18:1ω6c).

The type species is Notoacmeibacter marinus.

### DESCRIPTION OF NOTOACMEIBACTER MARINUS SP. NOV.

Notoacmeibacter marinus sp. nov. (mar.in ‘nus. L. masc. adj. marinus of the sea, isolated from the gut of a marine limpet, Notoacmea schrenckii on the intertidal rocks on Xiamen coast, China).

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**Table 1.** Phenotypic properties of strain XMTR2A4T and its close relatives

<table>
<thead>
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<th>Characteristic</th>
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<td>Isolation source*</td>
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<td>Seawater</td>
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<td>Trisodium citrate</td>
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<td>Malic acid</td>
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*Data from: a, Urakami et al. [9]; b, Mergaert et al. [11]; c, Peix et al. [10]; d, Scholla and Elkan [12]; e, Lai et al. [13].
†Symbol A represents leaf nodules of tropical ornamental plants (species of Myrsinaceae and Rubiaceae) and on the phylloplane and rhizoplane of other plants.
The species displays the following characteristics in addition to those given in the genus description. Cells are short rods, 2.0×0.7 μm and lack flagellum. Growth is observed at temperatures of 15 to 37 °C (optimum 28–30 °C), salinity range from 0.5 to 9 % (optimum 1–2 %), and pH 6 to 9 (optimum pH 7). Positive for alkaline phosphatase, acid phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, trypsin, and α-glucosidase activities; weakly positive for valine arylamidase, cysteine arylamidase, α-chymotrypsin, naphthol-AS-Bl-phosphohydrolase and urease activities. Nitrates can be reduced to nitrite. Fermentation of D-glucose is weak. D-Glucose, D-mannitol and malic acid can be utilized as sole carbon sources. The polar lipids consisted of phosphatidylethanolamine, phosphatidylcholine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylmonomethylethanolamine, an unidentified phospholipid and three unknown lipids.

The type strain is XMTR2A4\textsuperscript{T} (=MCCC 1A01882\textsuperscript{T}=KCTC 52427\textsuperscript{T}), isolated from the gut of a marine limpet, Notoacmea schrenckii. The G+C content of the type strain is 61.5 mol% calculated from draft genome assembly.

### DESCRIPTION OF NOTOACMEIBACTERACEAE

**FAM. NOV.**

Notoacmeibacteraceae (No.to.ac.me.i.bac.te.ra.ce’ae. N.L. masc. n. Notoacmeibacter a bacterial genus; -aceae ending to denote a family; N.L. fem. pl. n. Notoacmeibacteraceae the Notoacmeibacter family).

This family is affiliated with the order Rhizobiales in the class Alphaproteobacteria. The description is as the same as that for the genus Notoacmeibacter.

### Funding information

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### Conflicts of interest

The authors declare that there are no conflicts of interest.

### References