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TAXONOMIC DESCRIPTION

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Zobellia roscoffensis sp. nov. and *Zobellia nedashkovskayae* sp. nov., two flavobacteria from the epiphytic microbiota of the brown alga *Ascophyllum nodosum*, and emended description of the genus *Zobellia*

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Abstract

Four marine bacterial strains were isolated from a thallus of the brown alga *Ascophyllum nodosum* collected in Roscoff, France. Cells were Gram-stain-negative, strictly aerobic, non-flagellated, gliding, rod-shaped and grew optimally at 25–30 °C, at pH 7–8 and with 2–4% NaCl. Phylogenetic analyses of their 16S rRNA gene sequences showed that the bacteria were affiliated to the genus *Zobellia* (family *Flavobacteriaceae*, phylum *Bacteroidetes*). The four strains exhibited 97.8–100% 16S rRNA gene sequence similarity values among themselves, 97.9–99.1% to the type strains of *Zobellia amurskyensis* KMM 3526^T and *Zobellia laminariae* KMM 3676^T, and less than 99% to other species of the genus *Zobellia*. The DNA G+C content of the four strains ranged from 36.7 to 37.7 mol%. Average nucleotide identity and digital DNA–DNA hybridization calculations between the new strains and other members of the genus *Zobellia* resulted in values of 76.4–88.9% and below 38.5%, respectively. Phenotypic, phylogenetic and genomic analyses showed that the four strains are distinct from species of the genus *Zobellia* with validly published names. They represent two novel species of the genus *Zobellia*, for which the names *Zobellia roscoffensis* sp. nov. and *Zobellia nedashkovskayae* sp. nov. are proposed with Asnod1-F08^T (RCC6906^T=KMM 6823^T=CIP 111902^T) and Asnod2-B07-B^T (RCC6908^T=KMM 6825^T=CIP 111904^T), respectively, as the type strains.

The genus *Zobellia* was proposed by Barbeyron *et al.* [1] and contains Gram-negative, aerobic and gliding bacteria that produce flexirubin-type pigments. At the time of writing, the genus *Zobellia* comprises five validly named species, *Zobellia galactanivorans*, *Zobellia uliginosa* [1], *Zobellia amurskyensis*, *Zobellia laminariae* and *Zobellia russellii* [2], all isolated from marine environments. *Z. galactanivorans*, a species isolated from the red alga *Delesseria sanguinea* (Hudson) J. V Lamouroux 1813, was chosen as the type species.

During a previous study of the surface microbiota of the brown alga *Ascophyllum nodosum* (Linnaeus) Le Jolis 1863, 324 bacterial strains were isolated [3]. The taxonomic position of four isolates was investigated in the present study using a polyphasic approach, including some genomic data deduced from their draft genome, whole-genome comparison using the average nucleotide identity (ANI) and dDDH (digital DNA–DNA hybridization) methods, and phenotypic and physiological analyses. Based on these results, we report the description of two novel species of the genus *Zobellia*, for which the names *Zobellia roscoffensis* sp. nov. and *Zobellia nedashkovskayae* sp. nov. are proposed.

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Keywords: *Flavobacteriaceae*; macroalgal microbiome; polysaccharide; *Zobellia roscoffensis*; *Zobellia nedashkovskayae*.
Abbreviations: ANI, average nucleotide identity; dDDH, digital DNA–DNA hybridization; GGDC, Genome-to-Genome Distance Calculator; ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining.
The Genbank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences are as follows: MW114830 for strain Asnod1-F08^T, MW114831 for strain Asnod2-B02-B, MW114832 for strain Asnod2-B07-B^T, MW114833 for strain Asnod3-E08-A.
The Genbank/ENA/DDBJ accession numbers for the genome sequences are as follows: JADDXT01 for strain Asnod1-F08^T, JADDXS01 for strain Asnod2-B02-B, JADDXR01 for strain Asnod2-B07-B^T, JADDXQ01 for strain Asnod3-E08-A.
Three supplementary tables and three supplementary figures are available with the online version of this article.

The isolates investigated in this study were obtained by sampling healthy *A. nodosum* plants collected in the English Channel from the foreshore (48° 43′ 36.07″ N, −3° 59′ 22.96″ W) in Roscoff (Brittany, France) at the end of March 2014 [3]. Strains Asnod1-F08^T, Asnod2-B02-B, Asnod2-B07-B^T and Asnod3-E08-A were isolated by swabbing algal surfaces with sterile cotton tips and then inoculating plates of marine agar 2216 (Difco).

For comparison, *Z. amurskyensis* KMM 3526^T (=CIP 108562^T) and *Z. laminariae* KMM 3676^T (=CIP 108563^T) [2] were purchased from the Collection de l'Institut Pasteur (France) and used as related type strains. Except for the temperature, pH and NaCl ranges of growth, *Z. amurskyensis* KMM 3526^T and *Z. laminariae* KMM 3676^T were studied in parallel with the four new strains for all phenotypic tests and for quinone, fatty acid and polar lipid analyses. All strains were routinely cultivated on ZoBell medium 2216 [4], either liquid or solidified with 1.5% (w/v) agar. Pure cultures were stored at −80 °C in the culture medium containing 20% (v/v) glycerol. All experiments were performed in triplicate. Growth was evaluated in ZoBell agar plates at 4, 9, 11, 12, 13, 15, 17, 18, 20, 22, 24, 30, 37, 40, 42, 45 and 48 °C. The optimal pH value for growth was determined at 25 °C in ZoBell broth with pH values adjusted by using 100 mm of the following buffers: MES for pH 5.0; Bis Tris for pH 5.5, 6.0, 6.5, 7.0 and 7.5; Tris for pH 8.0, 8.5 and 9.0; CHES for pH 9.5 and CAPS for pH 10.0, 10.5 and 11.0. The effect of NaCl on growth was determined at 25 °C and at pH 8 in ZoBell broth prepared with distilled water containing 0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 10, 12, 15, 17, 20 and 25% NaCl. To test the influence of other salts on growth, the same NaCl range was used in ZoBell broth prepared with artificial seawater without NaCl but containing 6.3 g l^{-1} $MgSO_4$, 4.2 g l^{-1} $MgCl_2$ and 0.7 g l^{-1} KCl.

Cell morphology and gliding motility were investigated on wet mounts of an exponential phase ZoBell broth culture at 25 °C, by using phase-contrast microscopy on a BX60 instrument (Olympus). The Ryu non-staining KOH method [5] was used to determine the Gram type. Production of flexirubin was assessed by flooding 4-day plate culture with 20% (w/v) potassium hydroxide followed by the observation of changes in colony colour from yellow to red or brown [6]. Colony iridescence was assayed on 3-day plate cultures on R2A (Reasoner's 2A) agar (Difco) using a Stemi 2000-C stereomicroscope (Zeiss) equipped with a KC15000 LCP light source and a 455170 polarizing analyzer.

Oxidase activity was assayed using small pieces of 3MM paper (Whatman) soaked in the reagent *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine dihydrochloride (bioMérieux). Catalase activity was assayed by mixing one colony from a ZoBell agar plate with a drop of hydrogen peroxide (3 %, v/v). Amylase activity was assayed on 0.2% (w/v) soluble starch ZoBell agar plates. DNase activity was detected on DNA agar (Difco) prepared with seawater. Amylase and DNase activities were revealed by flooding the plates with Lugol's solution or 1 M HCl, respectively. The degradation of Tween compounds (1 %, v/v) was assayed in ZoBell agar according to Smibert and Krieg [7]. Agarase, κ-carrageenase and ι-carrageenase activities were tested by inoculating ZoBell media solidified with (per litre): 15 g agar (Sigma-Aldrich, ref. A7002), 10 g κ-carrageenin (X-6913, Danisco) or 20 g ι-carrageenin (X-6905, Danisco) respectively. ZoBell medium solidified with alginate was made using the calcium carbonate/gluconolactone method [8]. Strains were considered positive when colonies liquefied or produced craters in the solidified substrate. Moreover, agarase activity was revealed by flooding the plates with Lugol's solution. Additional phenotypic characterizations were performed using API 20 E, API 20 NE, API 50CH and API ZYM strips according to the manufacturer's instructions (bioMérieux) except that API AUX and API 50 CHB/E media were adjusted to 2.5% NaCl. All strips were inoculated with cell suspensions in artificial seawater and incubated at 25 °C for 48 h. The ability to use carbohydrates as sole carbon and energy sources was also tested in marine minimal medium [9] containing 2 g l^{-1} of the following sugars (all from Sigma-Aldrich unless otherwise stated): D-glucose, D-galactose, D-fructose, L-fucose, D-mannose, L-arabinose, L-rhamnose, D-xylose, lactose, sucrose, maltose, raffinose, D-mannitol, glucomannan (Megazyme), galactan (arabic gum), galactomannan from carob seeds (Megazyme), arabinan (Megazyme), arabinoxylane (Megazyme), xylan, xyloglucan (Megazyme), amylopectin (Merck), pectin (from apple), lichenin (Megazyme), laminarin (Goëmar), agar, porphyrin (extracted from *Porphyra* sp.), κ-carrageenin (Danisco), ι-carrageenin (Danisco), λ-carrageenin (Danisco), acid alginic (Danisco), fucoidin from *Ascophyllum nodosum* (kindly provided by Algues et Mer) and ulvan (kindly provided by Pr. Bruno Moerschbacher, University of Münster, Germany).

Sensitivity to antibiotics was tested by the disc-diffusion method on ZoBell agar plates and using antibiotic discs (Bio-Rad) containing (µg per disc, unless otherwise stated): penicillin G (10 IU), ampicillin (10), carbenicillin (100), oxacillin (5), streptomycin (500), kanamycin (30), chloramphenicol (30), tetracycline (30), lincomycin (15), bacitracin (130), rifampicin (30), vancomycin (30), trimethoprime/sulfamethoxazole (1.25/23.75), colistin (50), gentamicin (15), neomycin (30), nalidixic acid (30), polymixin (50) and erythromycin (15). The effects of the antibiotics on cell growth were assessed after 48 h of incubation at 25 °C, and susceptibility was scored based on the diameter of the clear zone around the disc.

Analyses of respiratory quinones [10, 11], fatty acids [12, 13] and polar lipids [14, 15] were performed on freeze-dried cultures of strains Asnod1-F08^T, Asnod2-B02-B, Asnod2-B07-B^T, Asnod3-E08-A and of *Z. amurskyensis* KMM 3526^T and *Z. laminariae* KMM 3676^T grown at room temperature in ZoBell medium 2216, by Susanne Verbarg and Dr. Brian Tindall from the DSMZ Identification Service, Braunschweig, Germany.

Genomic DNA was extracted from strains Asnod1-F08^T, Asnod2-B02-B, Asnod2-B07-B^T and Asnod3-E08-A using the DNeasy UltraClean Microbial kit (Qiagen). Sequencing libraries were prepared using the Nextera XT DNA kit

(Illumina) and sequenced using Illumina MiSeq v3 PE300, resulting in 5312862 quality-filtered reads for Asnod1-F08^T, 5534396 for Asnod2-B02-B, 4493736 for Asnod2-B07-B^T and 2037244 for Asnod3-E08-A (Table S1, available in the online version of this article). Reads were assembled using SPAdes version 3.11 [16] and scaffolds were built using MeDuSa version 1.6 [17] using the closed genome of *Z. galactanivorans* Dsij^T as a backbone. *In silico* gap-filling was performed using GapCloser version 1.12 [18]. The final assemblies consisted of two scaffolds (six contigs) for Asnod1-F08^T, six scaffolds (11 contigs) for Asnod2-B02-B, three scaffolds (five contigs) for Asnod2-B07-B^T and nine scaffolds (13 contigs) for Asnod3-E08-A. Completeness was checked with CheckM version 1.0.11 [19] by searching for 571 lineage-specific marker genes.

The 16S rRNA gene sequence was amplified by PCR using pure genomic DNA as template and the bacteria-specific primer pair, 8F [20] and 1492R [21]. PCR reactions were typically prepared in a volume of 25 µl containing 10–100 ng template, 0.4 µm each specific primer, 250 µm each dNTP, 0.1 mg bovine serum albumin, 1× GoTaq buffer (Promega) and 1.25 U GoTaq DNA polymerase (Promega). PCR amplification was performed as previously described [22]. PCR products were purified using the Exostar kit according to the manufacturer's protocol (GE Healthcare) and sequenced using BigDye Terminator version 3.1 (Applied Biosystems) and an ABI PRISM 3130xl Genetic Analyzer automated sequencer (Applied Biosystems/Hitachi). Chargaff's coefficient (G+C content) of the genomic DNA of strains Asnod1-F08^T, Asnod2-B02-B, Asnod2-B07-B^T and Asnod3-E08-A was deduced from the draft genome sequence and expressed as the molar percentage of guanine+cytosine. The nucleotide sequences of the 16S rRNA gene deduced from the draft genomes were compared to those obtained by direct sequencing of PCR-amplified products, showing high identity for each strain (99.85% for Asnod1-F08^T, 99.71% for Asnod2-B02-B, 99.86% for Asnod2-B07-B^T and 99.93% for Asnod3-E08-A). Genome-extracted 16S rRNA sequences for the four new strains were aligned with sequences of the 16S rRNA genes from all valid species of the genus *Zobellia* and from some species of the closest genera using the software MAFFT version 7 with the L-INS-I strategy [23]. The alignment was then manually refined and phylogenetic analyses, using the neighbour-joining (NJ) [24], maximum-parsimony (MP) [25] and maximum-likelihood (ML) [26] methods, were performed using the MEGA 6 package [27]. The different phylogenetic trees were built from a multiple alignment of 41 sequences and 1435 positions. For the NJ algorithm, Kimura's two-parameter evolutionary model was used [28]. The ML tree was calculated using Kimura's two-parameter evolutionary model with a discrete Gamma distribution to model evolutionary rate differences among sites (four categories). This substitution model was selected through submission of the alignment to the online server IQ-TREE (http://iqtree.cibiv.univie.ac.at/) [29]. The MP tree was obtained using the subtree-pruning–regrafting algorithm [30]. Bootstrap analysis (1000 replicates) was performed to provide confidence estimates for the phylogenetic tree topologies [31]. A phylogenomic tree was reconstructed with FastTree MP based on a concatenated alignment of 2573 translated protein-coding genes from the core genome of *Zobellia* species (50% amino acid identity, 80% coverage) using custom scripts. Pairwise comparisons of 16S rRNA gene sequences were made by using the database EzBioCloud (www.ezbiocloud.net/identify) [32] and FASTA software [33]. Genomic relatedness was investigated by comparing the new isolates genome sequence with those of the type strains of other *Zobellia* species using ANI (http://jspecies.ribohost.com/jspeciesws/#analyse) [34–36] and dDDH via the Genome-to-Genome Distance Calculator 2.1 (GGDC; http://ggdc.dsmz.de/distcalc2.php) [37]. The dDDH results from GGDC analysis were obtained from the alignment method BLAST+ and formula 2 (sum of all identities found in HSPs/by overall HSP length; HSP: High-scoring segment pairs) for incomplete genome sequences [38, 39]. Exploration of carbohydrate active enzyme-coding genes in the genomes of strains Asnod1-F08^T, Asnod2-B02-B, Asnod2-B07-B^T, Asnod3-E08-A and comparison with *Z. amurskyensis* KMM 3526^T and *Z. laminariae* KMM 3676^T [40] was carried out via the online server Microscope from the French National Sequencing Centre (www.genoscope.cns.fr/agc/microscope/mage) [41] and the CAZy database (www.cazy.org) [42].

Phylogenetic analyses of 16S rRNA genes of species from a subset of the family *Flavobacteriaceae* showed that strains Asnod1-F08^T, Asnod2-B02-B, Asnod2-B07-B^T and Asnod3-E08-A belong to the genus *Zobellia* (Figs 1 and S1). The 16S rRNA genes from Asnod1-F08^T and Asnod2-B02-B were included in a clade containing the type strains of *Z. amurskyensis* KMM 3526^T and *Z. laminariae* KMM 3676^T while the 16S rRNA genes from Asnod2-B07-B^T and Asnod3-E08-A were distantly related to type strains of *Z. galactanivorans* Dsij^T and *Z. uliginosa* 553^T (Figs 1 and S1). The best pairwise comparison scores with 16S rRNA genes from strains Asnod1-F08^T and Asnod2-B02-B (1525 bp for both) were obtained with *Zobellia galactanivorans* Dsij^T and *Z. uliginosa* 553^T (99.0%; Table S2) and with *Z. galactanivorans* Dsij^T and *Z. uliginosa* 553^T (98.8%; Table S2), respectively. From the 16S rRNA sequences of Asnod2-B07-B^T and Asnod3-E08-A (1523 for both), the best scores were obtained with *Z. laminariae* KMM 3676^T (99.1%; Table S2) for both strains. The 16S rRNA gene sequence similarities between the four new strains and other *Zobellia* species were in the range of 97.4% with *Z. russellii* KMM 3677^T and 98.5% with *Z. amurskyensis* KMM 3526^T (Table S2). The draft genomes of strains Asnod1-F08^T, Asnod2-B02-B, Asnod2-B07-B^T and Asnod3-E08-A were sequenced. Completeness was estimated at 100% for the four strains (Table S1). The genomic sizes were between 4941018 (Asnod2-B07-B^T) and 5025849 nucleotides (Asnod3-E08-A) and the Chargaff's coefficients were between 36.7% for Asnod3-E08-A and 37.7% for Asnod2-B02-B (Table S1). Analysis of a phylogenomic tree based on 2573 genes from the core genome of sequenced *Zobellia* strains showed that Asnod2-B07-B^T and Asnod3-E08-A formed a clade with *Z. laminariae* KMM 3676^T, while Asnod1-F08^T and Asnod2-B02-B were distant from all other strains (Fig. S2).

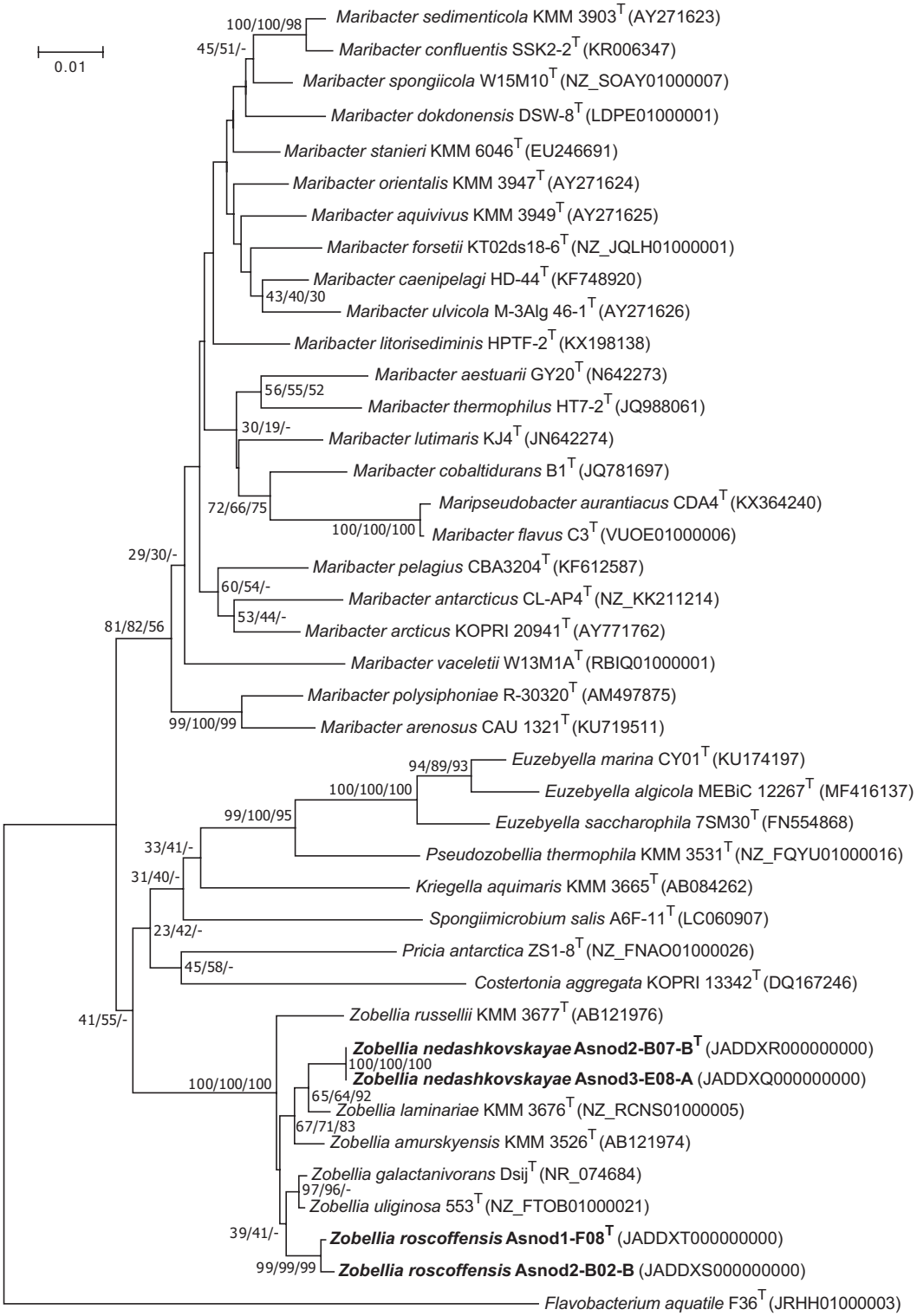

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences, showing the phylogenetic relationships between strains Asnod1-F08^T, Asnod2-B02-B, Asnod2-B07-B^T, Asnod3-E08-A and related taxa from the family *Flavobacteriaceae*. Numbers at the nodes indicate bootstrap values (in percentage of 1000 replicates) from neighbour-joining, maximum-likelihood and maximum-parsimony analyses respectively, while dashes instead of numbers indicate that the node was not observed in the corresponding analysis. For nodes conserved in at least two trees, all bootstrap values are shown. Nodes without bootstrap value are not conserved in other trees and <70%. *Flavobacterium aquatile* F36^T was used as an outgroup. Bar, 0.01 changes per nucleotide position.

ANI and dDDH results (formula 2) for the new isolates, when compared with the valid species of *Zobellia*, were less than 89% and less than 38.5%, respectively (88.9 and 38.3% with *Z. laminariae* KMM 3676^T; Table S3). As the normally accepted thresholds of species delineation for ANI and dDDH are 95 and 70%, respectively [34, 36, 43, 44], these values suggest that strains Asnod1-F08^T, Asnod2-B02-B, Asnod2-B07-B^T and Asnod3-E08-A do not belong to any of the valid species of the genus *Zobellia*. In addition, the genomes of the new isolates showed the highest ANI and dDDH similarities to *Z. laminariae* KMM 3676^T and *Z. amurskyensis* KMM 3526^T, suggesting that these two species were closest to the new isolates (Table S3). *Z. laminariae* KMM 3676^T and *Z. amurskyensis* KMM 3526^T were therefore studied in parallel with the novel isolates. Finally, the ANI and dDDH percentages between Asnod1-F08^T and Asnod2-B02-B (97.5 and 81.6% respectively) showed that these two strains represent a new species, for which the name *Zobellia roscoffensis* sp. nov. is proposed (Table S3). Similarly, the ANI and dDDH percentages between Asnod2-B07-B^T and Asnod3-E08 (97.6 and 82.1%, respectively) showed that these two strains represent a new species, for which the name *Zobellia nedashkovskayae* sp. nov. is proposed (Table S3).

Strains Asnod1-F08^T, Asnod2-B02-B, Asnod2-B07-B^T and Asnod3-E08-A cultivated on ZoBell medium 2216E showed yellow–orange colonies of 1 mm in diameter strongly attached to agar after 3 days at 25 °C. Flexirubin-type pigments were present in all four strains. A spreading aspect of colonies was observed only for strains Asnod1-F08^T and Asnod2-B02-B. A weak iridescence was observed only for Asnod1-F08^T and Asnod2-B02-B. The cells were Gram-stain-negative. Under the microscope, they appeared as rods attached to the glass of the slide or coverslip and showed gliding motility.

All four strains Asnod1-F08^T, Asnod2-B02-B, Asnod2-B07-B^T and Asnod3-E08-A used the non-gelling agaroid porphyrin as a sole carbon source (Table 1). Surprisingly, agar, although hydrolysed by strains Asnod1-F08^T, Asnod2-B02-B and *Z. amurskyensis* KMM 3526^T, is not used as sole carbon source by any of the strains including *Z. amurskyensis* KMM 3526^T and *Z. laminariae* KMM 3676^T (Table 1). This result might be explained by the presence of the gene *agaC* in the genomes of Asnod1-F08^T, Asnod2-B02-B and *Z. amurskyensis* KMM 3526^T only. An outer-membrane AgaC ortholog was recently characterized in *Z. galactanivorans* Dsij^T (GenBank accession CAZ98402) and shown to be an agarase active on complex agars [45]. The studied strains might lack one or several gene(s) for utilization of agar as sole carbon source, such as *agaA* (CAZ98338 in *Z. galactanivorans* Dsij^T), which is absent from their genomes. None of the tested carrageenins were hydrolysed or used as carbon and energy sources (Table 1). This observation is consistent with the absence of genes *cgkA* and *cgiA* encoding a κ- and ι-carrageenase, respectively, in the six studied genomes whereas the genome of the carrageenolytic *Z. galactanivorans* Dsij^T contains one *cgkA* (CAZ94309) and three *cgiA* genes (CAZ98400, CAZ96312, CAZ96032) [46]. Interestingly, all strains analysed in this study feature the complete operon responsible for the assimilation of 3,6-anhydro-D-galactose [47], a monosaccharide component of carrageenins. Although all *Zobellia* strains in this study possess between four and eight genes encoding alginate lyases, alginic acid was degraded and used as the sole carbon source by Asnod2-B07-B^T, Asnod3-E08-A and *Z. amurskyensis* KMM 3526^T only (Table 1). Furthermore, strains Asnod2-B07-B^T and Asnod3-E08-A liquefied the alginate while *Z. amurskyensis* KMM 3526^T only formed a crater. This difference in behaviour could be due to the absence of the gene *alyA1*, encoding a secreted endo-alginate lyase in *Z. galactanivorans* Dsij^T (CAZ95239) [48], in the genome of *Z. amurskyensis* KMM 3526^T, while *alyA1* is present in the genomes of strains Asnod2-B07-B^T and Asnod3-E08-A. Strains Asnod1-F08^T and Asnod2-B02-B did not degrade or utilize alginic acid as a unique carbon source (Table 1). This might be due to the absence of a gene encoding the alginate-specific SusD-like lipoprotein (CAZ96770 in *Z. galactanivorans* Dsij^T), involved in the binding and internalization of oligo-alginates [49]. Finally, it remains unexplained why *Z. laminariae* KMM 3676^T does not degrade alginic acid, since no difference in the alginate lyase gene content could be observed compared to *Z. amurskyensis* KMM 3526^T. Starch was hydrolysed and used as a sole carbon source by *Z. amurskyensis* KMM 3526^T, the only strain that showed a hydrolysis area on soluble starch ZoBell agar plates (Table 1). This result is consistent with the presence of the *susA* gene encoding an outer membrane α-amylase in its genome, while it is absent from strains Asnod1-F08^T, Asnod2-B02-B, Asnod2-B07-B^T, Asnod3-E08-A and *Z. laminariae* KMM 3676^T. Although all strains possess *malS*, encoding an α-amylase of the cytoplasmic membrane, *susA* might be necessary to use starch as unique carbon source. The other physiological features of Asnod1-F08^T, Asnod2-B02-B, Asnod2-B07-B^T and Asnod3-E08-A compared with *Z. amurskyensis* KMM 3526^T and *Z. laminariae* KMM 3676^T are listed in Table 1.

The six strains were resistant to penicillin, oxacillin, polymyxin B, bacitracin, colistin, gentamicin, neomycin, kanamycin, trimethoprime/sulfamethoxazole and nalidixic acid, and were sensitive to rifampicin. Asnod2-B07-B^T, Asnod3-E08-A, *Z. amurskyensis* KMM 3526^T and *Z. laminariae* KMM 3676^T were sensitive to lyncomycin and carbenicillin, while Asnod1-F08^T and Asnod2-B02-B were resistant to these antibiotics.

The major fatty acids (> 10% of the total fatty acids) of strains Asnod1-F08^T, Asnod2-B02-B, Asnod2-B07-B^T and Asnod3-E08-A were iso-$C_{15:0}$ (>20%), iso-$C_{17:0}$ 3-OH (>18%), iso-$C_{15:1}$ G (>10%) and those contained in summed feature 3 (>10%; $C_{16:1}$ ω7*c* and/or iso-$C_{15:0}$ 2OH) (Table 2). These fatty acid profiles were similar to those of *Z. amurskyensis* KMM 3526^T and *Z. laminariae* KMM 3676^T (Table 2) and confirmed previous results [2]. From all strains, the respiratory quinone was menaquinone-6 (MK-6). The major polar lipids of all strains were unidentified lipids and aminolipids and phosphatidylethanolamine (Fig. S3). *Z. amurskyensis* KMM 3526^T and *Z. laminariae* KMM 3676^T were characterized by the presence of two phosphatidylethanolamine spots. In conclusion, phenotypic characterizations and phylogenetic analyses

Table 1. Phenotypic characteristics of strains Asnod1-F08^T, Asnd2-B02-B, Asnod2-B07-B^T, Asnod3-E08-A and of two *Zobellia* species used as related type strains

Strains: 1, Asnod1-F08^T (*Z. roscoffensis* sp. nov.); 2, Asnod2-B02-B (*Z. roscoffensis* sp. nov.); 3, Asnod2-B07-B^T (*Z. nedashkovskayae* sp. nov.); 4, Asnod3-E08-A (*Z. nedashkovskayae* sp. nov.); 5, *Z. amurskyensis* KMM 3526^T; 6, *Z. laminariae* KMM 3676^T. Cells of all strains share the following characteristics: Gram-negative, aerobic, heterotroph, chemorganotroph with respiratory metabolism, gliding motility, do not form endospores, do not accumulate poly-*β*-hydroxybutyrate as an intracellular reserve product; require Na^+ ion or seawater for growth. All strains are positive for flexirubin production and nitrate reduction; for the utilization as a sole carbon source of D-glucose, D-galactose, D-fructose, rhamnose, sucrose, maltose, lactose, L-arabinose, mannose, xylose, mannitol and porphyrin; for the hydrolysis of DNA, aesculin and Tween 20; for acid and alkaline phosphatase, esterase lipase (C8), leucine, valine and cystine arylamidases, trypsin, α- and *β*-glucosidase, *β*-galactosidase (PNPG test), oxidase and catalase activities; for the acid production from D-glucose, D-galactose, D-fructose, mannose, rhamnose, D-xylose and maltose. All strains are negative for indole production; for utilization of caprate, adipate, citrate, gluconate, malate, phenylacetate, glucomannan, galactomannan, arabinan, xyloglucan, agar, κ-, ι- and λ-carrageenin and pectin; for the hydrolysis of κ- and ι-carrageenin; for urease, lipase (C14) and α-fucosidase activities; for the acid production from L-sorbose, melezitose, L-xylose, methyl-D-xylopyranoside, adonitol, L-arabitol, dulcitol, erythritol, xylitol, gentiobiose, raffinose, inulin, starch, glycogen, gluconate, 2-ketogluconate and 5-ketogluconate. +, Positive; –, negative; W, weakly positive; NA, not available.

Characteristic	1	2	3	4	5	6
Growth conditions:						
Optimum temperature (°C)	25–30	25–30	25–30	25–30	23–25*	21–23*
Temperature range (°C)	4–40	4–37	4–40	4–40	4–32*	4–30*
Optimum pH	7.5	7.5	7.5	7.5	NA	NA
pH range	5.5–8.5	5.5–8.5	5.5–8.0	5.5–8.0	NA	NA
NaCl range (%)	2–6	2–6	3–6	3–4	1–6*	1.5–6*
Optimum NaCl (%)	2	2	2	2	2*	2*
Iridescence	W	W	–	–	–	–
Enzyme (API 20 NE):						
Arginine dihydrolase	–	–	–	–	+	–
Gelatinase	–	+	–	–	+	+
Hydrolysis of:						
Starch (Lugol assay)	–	–	–	–	+	–
Agar (Lugol assay)	+	+	–	–	+	–
Alginic acid	–	–	+	+	+	–
Tween 40	–	–	+	+	–	–
Tween 60	+	–	+	+	+	–
Tween 80	+	+	+	–	–	–
Acid production:						
Glycerol	–	–	–	–	+	+
D-Arabinose	+	+	–	–	+	+
L-Arabinose	–	–	+	+	+	+
D-Ribose	–	–	–	–	+	+
Inositol	–	–	–	–	+	+
D-Mannitol	–	–	+	+	+	+
D-Sorbitol	–	–	–	–	+	+
Methyl-D-mannopyranoside	+	+	+	+	+	–
Methyl-D-glucopyranoside	+	+	+	+	+	–
N-Acetyl-glucosamine	+	+	+	+	–	–

Continued

Table 1. Continued

Characteristic	1	2	3	4	5	6
Amygdalin	+	+	+	+	+	−
Arbutin	+	+	+	+	−	−
Salicin	+	+	+	+	−	−
Cellobiose	+	+	+	+	w	−
Lactose	+	+	+	+	+	−
Melibiose	+	+	−	−	+	+
Sucrose	+	+	+	+	+	−
Trehalose	+	+	+	+	+	−
Turanose	+	+	+	+	+	−
D-Lyxose	+	+	+	+	+	−
D-Tagatose	+	+	−	−	+	−
D-Fucose	−	−	−	−	+	+
L-Fucose	+	+	−	−	+	+
D-Arabitol	−	−	−	−	+	−
Utilization of:						
N-Acetyl-glucosamine	+	+	−	−	+	+
L-Fucose	w	+	w	w	+	−
Raffinose	+	+	+	−	+	+
Amylopectin	−	−	−	−	+	−
Laminarin	−	−	+	+	+	w
Lichenin	w	w	−	−	w	w
Arabinoxylan	−	−	−	−	+	−
Fucoidin	−	−	+	+	+	w
Xylan	+	+	−	−	+	−
Ulvan	w	w	w	+	+	w
Alginic acid	−	−	+	+	w	w
Enzymes (API ZYM):						
α-Chymotrypsin	−	−	+	+	−	−
Naphthol phosphohydrolase	+	+	w	+	+	+
α-Galactosidase	−	+	−	−	+	−
β-Glucuronidase	−	−	−	−	−	−
N-Acetyl-*β*-glucosaminidase	−	+	−	−	+	+
α-Mannosidase	−	+	−	−	+	−
DNA G+C (mol%)	37.6	37.7	36.8	36.7	38.1	36.8

*Data from Nedashkovskaya [2].

Table 2. Cellular fatty acid composition of *Zobellia* species studied

Strains: 1, Asnod 1-F08^T (*Z. roscoffensis* sp. nov.); 2, Asnod2-B02-B (*Z. roscoffensis* sp. nov.); 3, Asnod2-B07-B^T (*Z. nedashkovskayae* sp. nov); 4, Asnod3-E08-A^T (*Z. nedashkovskayae* sp. nov); 5, *Z. amurskyensis* KMM 3526^T; 6, *Z. laminariae* KMM 3676^T. Data are percentages of the total fatty acids. Fatty acids that represented <1.0% in the six strains are omitted. Fatty acids that represented >10.0% are indicated in bold. –, Not detected or below 1%.

Fatty acid	1	2	3	4	5	6
Straight-chain:						
$C_{14:0}$	1.0	1.0	–	–	1.5	1.0
$C_{15:0}$	7.5	8.0	8.6	9.3	6.5	**10.3**
$C_{15:1}$ ω6*c*	1.0	1.0	1.7	1.8	1.2	1.1
$C_{16:0}$	–	1.0	–	–	–	1.0
$C_{16:0}$ 3-OH	1.2	1.0	1.1	1.0	1.6	1.4
$C_{17:1}$ ω6*c*	–	–	1.0	1.0	–	–
$C_{18:1}$ ω5*c*	–	–	1.5	1.4	1.0	1.0
Branched chain:						
iso-$C_{15:0}$	**26.7**	**25.2**	**20.9**	**22.6**	**29.5**	**25.6**
iso-$C_{15:0}$ 3-OH	3.2	3.1	3.6	3.4	3.0	2.8
anteiso-$C_{15:0}$	–	–	1.3	1.4	1.7	1.5
iso-$C_{15:1}$ G	**12.9**	**12.2**	**10.7**	**12.3**	**11.8**	9.4
iso-$C_{17:0}$ 3-OH	**19.8**	**20.3**	**21.2**	**18.8**	**15.4**	**18.7**
iso-$C_{17:1}$ ω9*c*	6.3	7.3	7.9	7.7	4.7	5.6
Summed features:*						
3	**10.7**	**10.9**	**13.8**	**12.9**	**14.9**	**14.2**
4	1.0	1.0	1.0	1.0	1.0	–
ECL:†						
13.565	2.7	2.8	1.4	1.3	1.6	1.4
16.582	1.2	1.2	1.2	1.2	1.0	1.0

*Summed features are fatty acids that cannot be resolved reliably from another fatty acid using the chromatographic conditions chosen. The MIDI system groups these fatty acids together as one feature with a single percentage of the total; summed feature 3 contained $C_{16:1}$ *ω7c* and/or iso-$C_{15:0}$ 2OH; summed feature 4 contained iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B.
†ECL, Equivalent chain-length. The identity of the fatty acid is not known

using 16S rRNA gene sequences and protein sequences from the core genomes of the genus *Zobellia* together with whole-genome pairwise comparisons show that strains Asnod1-F08^T, Asnod2-B02-B, on one hand, and Asnod2-B07-B^T and Asnod3-E08-A, on the other, represent two novel species in the genus *Zobellia*, for which the names *Zobellia roscoffensis* sp. nov. and *Zobellia nedashkovskayae* sp. nov. are proposed, respectively.

EMENDED DESCRIPTION OF THE GENUS *ZOBELLIA* BARBEYRON *ET AL.* 2001

The description of the genus is as given by Barbeyron *et al.* [1] with the following modified features. The hydrolysis of galactans from red seaweeds such as agar, κ- carrageenin and ι-carrageenin and of alginic acid from brown seaweeds depends on the species. The G+C content of the genomic DNA ranges from 36.7 to 43 mol%.

DESCRIPTION OF *ZOBELLIA ROSCOFFENSIS* SP. NOV.

Zobellia roscoffensis (ros.coff.en′sis. N.L. fem. adj. *roscoffensis* referring to Roscoff in Brittany, France, where the type strain was isolated).

Cells are Gram-stain-negative, aerobic, chemoorganotrophic, heterotrophic and rod-shaped, approximately 0.5 µm in diameter and 2.0–4.0 µm long; a few cells greater than 4 µm long may occur. Flagella are absent. Prosthecae and buds are not produced. Colonies on ZoBell agar are orange-coloured, convex, circular and mucoid in consistency and 2.0–3.0 mm in diameter strongly attached to agar after incubation for 3 days at 25 °C. A weak iridescence is visible on R2A agar after 3 days at 18 °C. Growth in ZoBell broth occurs from 4 to 40 °C (optimum, 25 °C), at pH 5.5–8.5 (optimum, pH 7.5) and in the presence of 2–6% NaCl (optimum, 2%). In the presence of magnesium and KCl, growth also occurs with 0 and 1% NaCl. Positive for gliding motility and flexirubin-type pigment production. Nitrate is reduced. *β*-Galactosidase-, oxidase- and catalase-positive. DNA, aesculin, Tweens 20, 60 (for the type strain only) and 80 and agar are hydrolysed, but Tween 40, gelatin (for the type strain only), starch, κ-carrageenin, ι-carrageenin and alginic acid are not. D-Glucose, D-galactose, D-fructose, rhamnose, *N*-acetyl-glucosamine, L-fucose (weakly for the type strain), raffinose, sucrose, maltose, lactose, L-arabinose, mannose, xylose, mannitol, xylan and porphyrin are utilized as carbon and energy sources, but caprate, adipate, citrate, gluconate, malate, phenylacetate, glucomannan, galactomannan, arabinan, arabinoxylan, xyloglucan, laminarin, amylopectin, agar, κ-, ι- and λ-carrageenin, ulvan, fucoidin (*Ascophyllum nodosum*), alginic acid and pectin (apple) are not. Acid is produced from D-glucose, D-galactose, D-fructose, mannose, D-arabinose, rhamnose, L-fucose, D-tagatose, D-lyxose, methyl-D-mannopyranoside, methyl-D-glucopyranoside, *N*-acetyl-glucosamine, amygdalin, arbutin, salicin, cellobiose, lactose, melibiose, sucrose, trehalose, turanose and maltose, but not from L-arabinose, D-ribose, D-xylose, L-sorbose, melezitose, L-xylose, D-fucose, methyl-D-xylopyranoside, glycerol, inositol, D-mannitol, D-sorbitol, adonitol, L- and D-arabitol, dulcitol, erythritol, xylitol, gentiobiose, raffinose, inulin, starch, glycogen, gluconate, 2-ketogluconate and 5-ketogluconate. Negative for indole and H_2S production and for arginine dihydrolase, tryptophan deaminase, urease, lysine decarboxylase and ornithine decarboxylase activities. In the API ZYM system, activities from acid phosphatase, alkaline phosphatase, esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase,

trypsin, α-glucosidase, *β*-glucosidase and naphthol-AS-BI-phosphohydrolase are present, but activities of lipase (C14), α-chymotrypsin, *β*-glucuronidase and α-fucosidase are absent. The only lipoquinone detected is MK-6. The major fatty acids (> 10% of the total fatty acids) are iso-$C_{15:0,}$ iso-$C_{17:0}$ 3-OH, iso-$C_{15:1}$ G and summed feature 3 ($C_{16:1}$ ω7*c* and/or iso-$C_{15:0}$ 2OH). The major polar lipids are phosphatidylethanolamine and unknown aminolipids. The DNA G+C content is 37.6–37.7 mol%.

The type strain, Asnod1-F08^T (RCC6906^T=KMM 6823^T=CIP 111902^T), and strain Asnod2-B02-B (RCC6907=KMM 6824=CIP 111903) were isolated from *Ascophyllum nodosum* surface microbiota.

DESCRIPTION OF *ZOBELLIA NEDASHKOVSKAYAE* SP. NOV.

Zobellia nedashkovskayae (ne.dash.kovs'ka.yae N.L. gen. n. *nedashkovskayae* in honour of Olga Nedashkovskaya for her great contribution to the study of marine *Bacteroidetes*).

Cells are Gram-stain-negative, aerobic, chemoorganotrophic, heterotrophic and rod-shaped, approximately 0.5 µm in diameter and 2.0–4.0 µm long; a few cells greater than 4 µm long may occur. Flagella are absent. Prosthecae and buds are not produced. Colonies on ZoBell agar are orange yellow-coloured, convex, circular and mucoid in consistency and 2.0–3.0 mm in diameter strongly attached to agar after incubation for 3 days at 25 °C. Growth in ZoBell broth occurs from 4–40 °C (optimum, 25–30 °C), at pH 5.5–8.0 (optimum, pH 7.5) and in the presence of 3–6% NaCl (optimum, 2%). In presence of magnesium and KCl, the growth also occurs with 1% NaCl. Positive for gliding motility and flexirubin-type pigment production. Nitrate is reduced. *β*-Galactosidase-, oxidase- and catalase-positive. DNA, aesculin, Tweens 20, 40, 60 and 80 (for the type strain only) and alginic acid are hydrolysed, but gelatin, starch, agar, κ-carrageenin and ι-carrageenin are not. D-Glucose, D-galactose, D-fructose, rhamnose, raffinose, sucrose, maltose, lactose, L-arabinose, mannose, xylose, mannitol, laminarin, porphyrin, fucoidin (*Ascophyllum nodosum*) and alginic acid are utilized as carbon and energy sources, but L-fucose, *N*-acetyl-glucosamine, caprate, adipate, citrate, gluconate, malate, phenylacetate, glucomannan, galactomannan, arabinan, arabinoxylan, xyloglucan, xylan, amylopectin, agar, κ-, ι- and λ-carrageenin, ulvan and pectin (apple) are not. Acid is produced from D-glucose, D-galactose, D-fructose, mannose, L-arabinose, rhamnose, D-xylose, D-lyxose, methyl-D-mannopyranoside, methyl-D-glucopyranoside, *N*-acetyl-glucosamine, D-mannitol, amygdalin, arbutin, salicin, cellobiose, lactose, sucrose, trehalose, turanose and maltose, but not from D-arabinose, D-ribose, L-sorbose, melezitose, L-xylose, D-fucose, L-fucose, D-tagatose, methyl-D-xylopyranoside, glycerol, inositol, D-sorbitol, adonitol, L- and D-arabitol, dulcitol, erythritol, xylitol, melibiose, gentiobiose, raffinose, inulin, starch, glycogen, gluconate, 2-ketogluconate and 5-ketogluconate. Negative for indole and H_2S production and for arginine dihydrolase, tryptophan deaminase, urease, lysine decarboxylase and ornithine decarboxylase activities. In the API ZYM system, activities from acid phosphatase, alkaline phosphatase, esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-glucosidase, *β*-glucosidase and naphthol-AS-BI-phosphohydrolase are present, but activities of lipase (C14), *β*-glucuronidase, α-galactosidase, *N*-acetyl-*β*-glucosaminidase and α-fucosidase are absent. The only lipoquinone detected is MK-6. The major fatty acids (>10% of the total fatty acids) are iso-$C_{15:0}$, iso-$C_{17:0}$ 3-OH, iso-$C_{15:1}$ G and summed feature 3 ($C_{16:1}$ ω7*c* and/or iso-$C_{15:0}$ 2OH). The major polar lipids are phosphatidylethanolamine and unknown aminolipids. The DNA G+C content is 36.7–36.8 mol%.

The type strain, Asnod2-B07-B^T (RCC6908^T=KMM 6825^T=CIP 111904^T), and strain Asnod3-E08-A (RCC6909=KMM 6826=CIP 111905) were isolated from *Ascophyllum nodosum* surface microbiota.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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