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TAXONOMIC DESCRIPTION

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Leeuwenhoekiella aestuarii sp. nov., isolated from salt-water sediment and first insights in the genomes of *Leeuwenhoekiella* species

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Abstract

Four Gram-negative, aerobic, rod-shaped and yellow-orange pigmented bacteria (R-46770, R-48165^T, R-50232 and R-50233) were isolated from intertidal sediment and water of the Westerschelde estuary between 2006 and 2012. Analysis of their 16S rRNA gene sequences revealed that the four strains form a separate cluster between validly described type strains of the genus *Leeuwenhoekiella*. DNA–DNA reassociation values of two representative strains (i.e. R-48165^T and R-50232) of the new group with type strains of *Leeuwenhoekiella* species ranged from 18.7 to 56.6%. A comparative genome analysis of the two strains and the type strains confirmed average nucleotide identity values from 75.6 to 94.4 %. The G+C contents of the genomic DNA of strains R-48165^T and R-50232 were 37.80 and 37.83mol%, respectively. The predominant cellular fatty acids of the four novel strains were summed feature 3 (i.e. $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH), iso-$C_{15:0}$, iso-$C_{15:1}$ G and iso-$C_{17:0}$ 3-OH. The four new *Leeuwenhoekiella*-like strains grew with 0.5–12% (w/v) NaCl, at pH 5.5–9.0 and displayed optimum growth between 20 and 30 °C. Based on the results of phenotypic, genomic, phylogenetic and chemotaxonomic analyses, the four new strains represent a novel species of the genus *Leeuwenhoekiella* for which the name *Leeuwenhoekiella aestuarii* sp. nov. is proposed. The type strain is LMG 30908^T (=R-48165^T=CECT 9775^T=DSM 107866^T). Genome analysis of type strains of the genus *Leeuwenhoekiella* revealed a large number of glycosyl hydrolases, peptidases and carboxyl esterases per Mb, whereas the number of transporters per Mb was low compared to other bacteria. This confirmed the environmental role of *Leeuwenhoekiella* species as (bio) polymer degraders, with a specialization on degrading proteins and high molecular weight compounds. Additionally, the presence of a large number of genes involved in gliding motility and surface adhesion, and large numbers of glycosyl transferases per Mb confirmed the importance of these features for *Leeuwenhoekiella* species.

INTRODUCTION

The genus *Leeuwenhoekiella* Nedashkovskaya *et al.* 2005 emend Nedashkovskaya *et al.* 2014 [1, 2] was originally proposed to accommodate Gram-negative, strictly aerobic, yellow-pigmented, chemo-organotrophic bacteria with a gliding motility [1]. At present, the genus comprises six validly described species [1–6]. Most of these strains were isolated from open ocean surface seawater. Nevertheless, cultivated *Leeuwenhoekiella* strains, metagenome-assembled *Leeuwenhoekiella* genomes and environmental sequences highly similar to *Leeuwenhoekiella* species have been obtained, sometimes in high abundance, from many other, predominantly marine sources worldwide [7–15]. Several studies have shown the presence of this taxon in deep-seawater samples and sediment, Arctic and Antarctic ice, the rhizosphere of halophytes and in hydrothermal plumes. Additionally, their presence in soft coral, sea urchin and as helper bacteria in co-cultures of *Prochlorococcus*, together with their ability to

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Keywords: *Leeuwenhoekiella*; diatom; intertidal sediment.
Abbreviations: ANI, average nucleotide identity; CE, carboxyl esterase; CRISPR, clustered regularly interspaced short palindromic repeat; GH, glycosyl hydrolase; *is*DDH, *in silico* DNA–DNA hybridization; MA, marine agar; MALDI-TOF MS, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PL, polysaccharide lyase; PUL, polysaccharide utilization loci.
Raw sequence data are available from the NCBI sequence read archive (https://www.ncbi.nlm.nih.gov/sra) under accession number SRP155464. The annotated Whole Genome Shotgun projects of strains LMG 30908T (R-48165^T=CECT 9775^T=DSM 107866^T), LMG 30909 (R-50232), LMG 1345^T, LMG 22550^T, LMG 24856^T and LMG 29608^T have been deposited at DDBJ/ENA/GenBank under the accession numbers QOVJ00000000, QOVI00000000, QOVL00000000, QOVM00000000, QOVN00000000 and QOVK00000000, respectively. The complete 16S rRNA gene sequences of strains LMG 30908T and LMG 30909 have been deposited at DDBJ/ENA/GenBank under the accession numbers MK433602 and MK433603, respectively.
One supplementary figure and five supplementary tables are available with the online version of this article.

hydrolyse diverse amides, esters and glucosides, as well as use various labile carbon sources, may indicate *Leeuwenhoekiella* species play important roles in the marine food web, potentially by providing growth factors and/or removing inhibitory factors [1, 4, 11, 16].

As part of several studies on diatom-associated bacteria present in intertidal sediments and the water of the Westerschelde estuary, The Netherlands, a number of bacterial isolates were obtained over several years. Preliminary identification using partial 16S rRNA gene sequences indicated that four of them (i.e. strains R-46770, R-48165^T, R-50232 and R-50233), isolated from intertidal sediments or from non-axenic cultures of the diatom *Cylindrotheca* cf. *fusiformis* originating from the same location have near-identical 16S rRNA gene sequences related to the genus *Leeuwenhoekiella*. Strains R-46770 and R-50232 were among 35 estuarine bacterial isolates included in a study investigating the influence of diatom-associated bacteria on the growth *Cylindrotheca closterium* (Stock *et al.* 2019, Submitted). Co-cultivation experiments with *C. closterium* WS3_7 showed that both strains significantly reduced the growth of *C. closterium* WS3_7, compared to the axenic control (Stock *et al*. 2019, Submitted). In this study, the novel *Leeuwenhoekiella*-like isolates were taxonomically characterized using a polyphasic approach and comparative genomics. Based on the results, we propose that the four strains represent a novel species of the genus *Leeuwenhoekiella*: *Leeuwenhoekiella aestuarii*.

METHODS

Isolation

Strain R-46770 was isolated in 2011 from a non-axenic culture of the diatom *Cylindrotheca* cf. *fusiformis* DCG 0423, which had been obtained from a water sample collected in January 2006 from the Westerschelde estuary, The Netherlands (51° 21′ N, 3° 43′ E). Strains R-50232 and R-50233 were isolated in 2012 from another non-axenic diatom culture containing *Cylindrotheca* cf. *fusiformis* DCG 0424, which had also been obtained from a water sample collected in January 2006 from the Westerschelde estuary, The Netherlands (51° 19′ N, 4° 16′ E). Strain R-48165^T was isolated in 2010 from sediment collected in the Paulina salt marsh in the same estuary (51° 21′ N, 3° 43′ E) earlier that year. Strain R-46770 was isolated on marine agar (MA) supplemented with 2 g l^{-1} glucose and galactose. The other strains were isolated on MA. After primary isolation and purification, all strains were stored at −80 °C in marine broth 2216 (Difco) supplemented with 15% (v/v) glycerol.

MALDI-TOF MS profiling

For acquisition of a matrix-assisted laser desorption/ionization time-of flight mass spectrometry (MALDI-TOF MS) profile, the four strains as well as type strains of the six validly described *Leeuwenhoekiella* species were grown on MA. Resuscitated cultures were subcultured twice over a time period of 1 week. Preparation of cell extracts and acquisition of bacterial fingerprints were performed according to Wieme *et al.* [17]. Subsequently, raw mass spectra were extracted as t2d files and converted into text files using the Data Explorer 4.0 software (Applied Biosystems). The text files were then imported in BioNumerics 7.5 (Applied Maths) and transformed into fingerprints. Finally, the similarity between fingerprints was determined using Pearson's product moment correlation after which spectra were clustered using unweighted pair group method with arithmetic mean.

PCR-based 16S rRNA gene analysis

For determination of the near-complete 16S rRNA gene sequence, DNA was prepared using the alkaline lysis protocol [18]. Amplification and sequencing of the 16S rRNA gene was performed as previously described by Tahon and Willems [19].

Genome sequencing and analyses

To allow genome-based analyses, the genomes of strains R-48165^T (=LMG 30908^T=CECT 9775^T=DSM 107866^T) and R-50232 (=LMG 30909) were sequenced. Additionally, *Leeuwenhoekiella* type strains for which no genomic sequence was available were sequenced (i.e. *Leeuwenhoekiella marinoflava* LMG 1345^T, *Leeuwenhoekiella palythoae* LMG 24856^T, *Leeuwenhoekiella aequorea* LMG 22550^T and *L. polynyae* LMG 29608^T). Genomic sequences for *L. blandensis* MED 217^T (=LMG 28563^T) and *Leeuwenhoekiella nanhiaensis* G18^T (=LMG 30890^T) were already publicly available. For sequencing, genomic DNA was extracted using an automated Maxwell DNA preparation instrument (Promega) as detailed by Tahon *et al.* [20]. Subsequently, the genomic sequences were determined using the Illumina HiSeq 2500 platform with 2×125 bp cycles at Baseclear. The genomes were assembled using Shovill 0.9.0 (https://github.com/tseemann/shovill). For the trimming step using Trimmomatic [21], the default Shovill options were changed so that only reads of which all positions had a Phred score of Q30 or higher were retained. The QUAST program was used to generate the summary statistics of the assembly (e.g. N50, G+C content) [22]. Contigs smaller than 500 bp were discarded. Final contigs were submitted for genome annotation using the Integrated Microbial Genomes–Expert Review (IMG-ER) platform [23] and the RAST server [24].

Identification and annotation of prophage sequences within the genome was performed using PHASTER [25]. Presence of clustered regularly interspaced short palindromic repeats (CRISPRs) was analysed using CRISPRCasFinder [26]. Identification of glycosyl hydrolases (GHs), glycosyl transferases, polysaccharide lyases (PLs) and carbohydrate esterases (CEs) was performed using the CAZy database [27]. Presence and identification of peptidases was performed using the MEROPS database [28]. Transporters were identified using TransAAP [29]. Sulfatases were identified using SulfAtlas [30].

Pairwise average nucleotide identity (ANI) was determined using the built-in tool in the IMG-ER platform [31]. *In silico* DNA–DNA hybridization (*is*DDH) was carried out using the Genome-to-Genome Distance Calculator 2.1 of DSMZ [32].

Determination of the core genome of the genus *Leeuwenhoekiella* was performed using Orthofinder [33] and the PATRIC server [34].

The annotated Whole Genome Shotgun projects have been deposited at DDBJ/ENA/GenBank (Table 1). Raw sequence data are available from the NCBI sequence read archive (www.ncbi.nlm.nih.gov/sra) under accession number SRP155464.

Phylogenetic analyses

For analysis of the 16S rRNA gene, the complete 16S rRNA gene sequences of all *Leeuwenhoekiella* type strains and several representatives of the family *Flavobacteriaceae* were extracted from the annotated genomes. The 16S rRNA gene sequence extracted from the genome of *Flexibacter flexilis* DSM 6793^T was used as an outgroup. Sequences were aligned using MEGA 6 [35]. Subsequently, a phylogenetic maximum-likelihood tree (1000 bootstraps) was reconstructed.

For a genome-based phylogeny, all *Leeuwenhoekiella* genomes and several representatives of the family *Flavobacteriaceae* were screened for the presence of 107 single-copy core genes, found in a majority of bacteria, using the automated bcgTree pipeline [36] with default parameters except that 1000 bootstraps were used. The genome of *Flexibacter flexilis* DSM 6793^T was used as an outgroup. Trees were visualized using the iTOL software [37].

Fatty acid analysis

For cellular fatty acid analysis, strains R-46770, LMG 30908^T, LMG 30909 and R-50233 and the six type strains were incubated on MA at 28 °C for 48 h. After fatty acid methyl-ester extraction, separation by gas-liquid chromatography was performed using the MIDI system (Microbial ID Inc.) as previously described [38]. Fatty acid methyl esters were identified by comparison to the MIDI Peak Library version 5.0.

Pigment extraction and spectral analysis

Single colonies of strains R-46770, LMG 30908^T, LMG 30909, R-50233 and type strains of the other validly published species of the genus *Leeuwenhoekiella* were grown on MA at 28 °C for 48 h. Pigment extraction was performed according to Henriques *et al.* [39] and Mohammedi *et al.* [40]. Briefly, two 10 µl loops of colony material were transferred to 1 ml physiological water (0.9% NaCl) and bacteria were harvested by centrifuge at 5000 ***g*** for 5 min. Subsequently, the supernatant was removed after which cells were resuspended in 2 ml of MilliQ and subjected again to centrifugation. This process was repeated twice. Afterwards, cells were resuspended in 1 ml methanol (≥99.9%; Sigma-Aldrich), strongly vortexed for 30 s and heated at 85 °C for 20 min. This mixture was centrifuged at 5000 ***g*** for 5 min to allow separation of the methanol extract from cells. This extraction process was repeated several times until the cells were completely bleached. Pigment extracts were characterized by spectrophotometry using a SPECTRAmax Plus 384 spectrophotometer (Molecular Devices). The absorbance spectrum was measured between 190 and 1000 nm. Subsequently, the samples were alkalinized with 0.1 M NaOH after which the spectra were examined for a bathychromatic shift characteristic of flexirubin-type pigments [41]. Additionally, pigment extracts were flooded with 20% KOH to examine presence of a colour shift indicating presence of a flexirubin-type pigment [42].

Morphology and metabolic profile

Cell morphology was observed using a phase-contrast microscope (Olympus BX40) after incubation on MA at 28 °C for 72 h. Gram staining was performed as previously described by MacFaddin [43]. Catalase activity was determined by bubble production in 3% (v/v) H_2O_2 and oxidase activity was determined using 1% (w/v) tetramethyl *p*-phenylenediamine. Gliding motility was evaluated using the hanging drop technique as described previously by Bernardet *et al.* [44]. Unless stated otherwise, metabolic profiling of the strains was performed at 28 °C in three replicates. Salt tolerance was tested by growing the strains in modified artificial seawater (0.13% KCL, 0.5% yeast extract, 1% peptone, 3% $MgCl_2{\cdot}6H_2O$, 0.05% NH_4Cl, 0.01% $CaCl_2{\cdot}2H_2O$, 0.014% KBr, 0.002% K_2HPO_4). Growth with 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 15 and 17% (w/v) NaCl was tested. Growth at different pH values (5.0–9.0 at intervals of 0.5 pH units) was assessed after growth in TYS broth (0.1% yeast extract, 0.5% tryptone, 2.75% NaCl, 0.54% $MgCl_2{\cdot}6H_2O$, 0.68% $MgSO_4{\cdot}7H_2O$, 0.1% KCl, 0.02% $NaHCO_3$, 0.14% $CaCl_2{\cdot}2H_2O$). Depending on the final pH needed, media were buffered with MES (pH 5.0–6.0), MOPS (pH 6.5–7.0), ACES (pH 7.5), TAPS (pH 8.0–8.5) or CHES (pH 9.0) in a final concentration of 5 mM. To determine pH and salt tolerance ranges and optima, growth was monitored by measuring the optical density three times at 600 nm after 72 h. Growth at 4, 10, 15, 20, 25, 28, 30, 37, 41 and 44 °C was determined on MA. Growth in anaerobic atmosphere (80% N_2, 10% CO_2 and 10% H_2, max 50 p.p.m. O_2) and growth in microaerobic atmosphere (80% N_2, 15% CO_2 and 5% O_2) was tested at 28 °C on MA. Antibiotic sensitivity was determined by growing the strains on MA and using antimicrobial susceptibility paper discs. The following antibiotics (Oxoid) were tested: gentamicin (10 µg), ampicillin (10 µg), tetracycline (30 µg), chloramphenicol (30 µg), bacitracin (10 µg) and vancomycin (30 µg).

Hydrolysis of carboxymethylcellulose was determined with 0.2% aqueous Congo red on MA containing 0.5% (w/v) carboxymethylcellulose. Hydrolysis of starch was tested with Lugol's iodine solution on MA supplemented with 0.5% (w/v) starch. Hydrolysis of casein was determined after culture on MA containing 3% (w/v) casein. For hydrolysis of DNA, two different approaches were used: (i) culture on DNAse Test Agar (Difco) containing 0.005% (w/v) methyl green. A change in colour around the bacterial growth indicates a positive reaction. (ii) Culture on DNAse Test Agar followed by flooding with 1M HCl. Here, a positive reaction is indicated by the appearance of a transparent halo around the colonies. Hydrolysis of Tweens 20, 40, 60 and 80 was determined after culturing on MA containing 1% of one of the Tweens. A cloudy halo around the bacterial colonies indicated positive activity. Carbon source utilization, acid formation from

Table 1. Characteristics of *Leeuwenhoekiella* genomes

Strains: 1, LMG 30908^T; 2, LMG 30909; 3, *Leeuwenhoekiella marinoflava* LMG 1345^T; 4, *Leeuwenhoekiella aequorea* LMG 22550^T; 5, *Leeuwenhoekiella palythoae* LMG24856^T; 6, *Leeuwenhoekiella blandensis* MED 217^T; 7, *Leeuwenhoekiella polynyae* LMG29608^T; 8, *Leeuwenhoekiella nanhaiensis* G18^T. TDM, thermal denaturation method, –, not performed.

Characteristic	1	2	3	4	5	6	7	8
Size (Mb)	4.43	4.36	4.74	3.46	3.94	4.24	4.79	4.36
Contigs	34	23	53	18	22	15	47	48
N50 (kb)	638	727	190	502	619	393	174	263
DNA G+C content (mol%)	37.80	37.83	37.52	35.41	39.96	39.76	38.11	42.10
DNA G+C content (mol%) (TDM)	–	–	38.00	35.00–36.00	41.20	42.50	38.80	35.00
Genes (total)	3859	3795	4138	3157	3374	3784	4138	3860
CDS (coding)	3805	3734	4065	3102	3326	3735	4075	3794
Genes (RNA)	54	61	73	55	48	49	63	66
5S rRNA	1	1	4	1	1	3	1	1
16S rRNA	1	1	1	1	1	2	1	1
23S rRNA	1	1	2	1	1	1	1	1
tRNA	36	36	40	38	36	43	36	36
Accession number	QOVJ00000000	QOVI00000000	QOVL00000000	QOVM00000000	QOVN00000000	AANC00000000	QOVK00000000	NQXA00000000
CRISPR (questionable)	0	1	0	2	0	3	1	2
CRISPR (high evidence)	0	0	0	0	1	1	1	0
Prophage region (incomplete)	0	0	0	0	2	1	3	1
Glycosyl hydrolases	95	95	98	47	97	103	119	98
Glycosyl transferases	30	27	26	30	27	31	32	27
Carboxyl esterases	16	16	16	8	16	13	16	15
Peptidases	161	161	158	139	153	164	168	158
Sulfatases	5	5	5	1	3	6	5	15
Transporters	257	255	271	218	234	240	268	251

carbohydrates and enzyme activity were determined using API ZYM, API 50 CH, API 20 NE (bioMérieux) and the GEN III MicroPlate (Biolog) systems. For the API 50 CH and API 20 NE assays, the manufacturer's protocols were changed as these did not result in growth or positive reactions. Because of the marine nature of the strains, inoculation fluids were modified to contain a final concentration of 2% NaCl and 10% marine broth. After inoculation, these galleries were incubated for 48 h at 28 °C before reactions were read. For the GEN III MicroPlate assay, the manufacturer's protocol was used except that, before use, 9 ml inoculation fluid B (IF-B) was supplemented with 1 ml of a 20% NaCl (w/v) solution. After inoculation, the galleries were incubated at 25 °C. Because the negative control frequently reacted and displayed a light purple colour, positive reactions were determined using a spectrophotometer (590 nm). The value of the negative control was subtracted from all other wells. Resulting values >0.075 were considered as positive.

Availability of biological material

The proposed type strain, R-48165^T, was deposited in the Belgian Co-ordinated Collections of Microorganisms (BCCM/LMG, Ghent, Belgium), the German Collection of Microorganisms and Cell Cultures (DSMZ) and the Spanish Type Culture Collection (CECT) under the accession numbers LMG 30908^T, DSM 107866^T and CECT 9775^T, respectively. Strain R-50232 was also deposited in the BCCM/LMG collection under the accession number LMG 30909. Strains R-46770 and R-50233 are stored in the research collection of the Laboratory of Microbiology at Ghent University and are available for further research.

RESULTS AND DISCUSSION

MALDI-TOF MS analysis

Clustering of the MALDI-TOF MS fingerprints showed that four strains (R-46770, LMG 30908^T, LMG 30909 and R-50233) formed a separate cluster in between type strain profiles from the six validly described *Leeuwenhoekiella* species (Fig. S1, available in the online version of this article). In this clustering, the similarity between the profiles of the four new strains and that of their closest neighbour, *Leeuwenhoekiella polynyae* LMG 29608^T, is similar to that between other members of the genus. This indicates that the four strains very likely represent a novel species in the genus *Leeuwenhoekiella*.

Phylogenetic placement and phylogenomics

Analysis of the near-complete 16S rRNA gene sequences of the four *Leeuwenhoekiella*-like strains showed that they were identical. Comparison with data in the EZBioCloud database (www.ezbiocloud.net/) gave *L. polynyae* LMG 29608^T as the closest neighbour with a similarity of 98.76% and *L. blandensis* MED 217^T as the most distant neighbour in the genus *Leeuwenhoekiella* with a similarity of 96.83% (Table 2). Additionally, phylogenetic maximum-likelihood analysis showed that the strains formed a robust cluster within the genus *Leeuwenhoekiella* (Fig. 1), indicating the four new strains could represent a new species in the genus *Leeuwenhoekiella*. To conduct a more robust analysis, two strains (i.e. LMG 30908^T and LMG 30909) were selected for whole genome sequencing.

Analysis of the complete 16S rRNA gene sequences of strains LMG 30908^T and LMG 30909 with those of type strains of the six validly described *Leeuwenhoekiella* species indicated that the sequence similarities ranged from 97.35% to *L. palythoae* LMG 24856^T to 99.02% to *L. marinoflava* LMG 1345^T. These results differed from those obtained using near-complete 16S rRNA gene sequences. In the case of *Leeuwenhoekiella*, PCR-based methods lead to sequences that are ~90 bp shorter than those derived from genomic data. This will, undoubtedly, influence and bias the similarity observations. For example, comparison of the PCR-based partial 16S rRNA gene sequence of LMG 30908^T and LMG 30909 with that of *L. marinoflava* LMG 1345^T results in a similarity of 98.68%, which is 0.34% lower than the similarity obtained using full 16S rRNA gene sequences (data not shown). When comparing this for all validly described *Leeuwenhoekiella* reference strains, differences up to 0.57% similarity can be observed (data not shown). It is well known that 16S rRNA gene analysis and the recommended threshold are often insufficient to discriminate between closely related species [45]. Our results suggest they can also be greatly influenced by the

Table 2. Similarity (%) between amplified near-complete 16S rRNA gene sequences of representative genomes of *Leeuwenhoekiella* species

Strains: 1, LMG 30908^T; 2, LMG 30909; 3, *Leeuwenhoekiella marinoflava* LMG 1345^T; 4, *Leeuwenhoekiella aequorea* LMG 22550^T; 5, *Leeuwenhoekiella palythoae* LMG24856^T; 6, *Leeuwenhoekiella blandensis* MED 217^T; 7, *Leeuwenhoekiella polynyae* LMG29608^T; 8, *Leeuwenhoekiella nanhaiensis* G18^T.

Strain	1	2	3	4	5	6	7	8
1		100.00	98.68	97.23	97.17	96.83	98.76	97.59
2			98.68	97.23	97.17	96.83	98.76	97.59
3				96.58	97.02	96.67	98.06	97.22
4					96.95	97.08	97.43	96.46
5						98.28	96.62	97.86
6							96.83	98.14
7								97.31

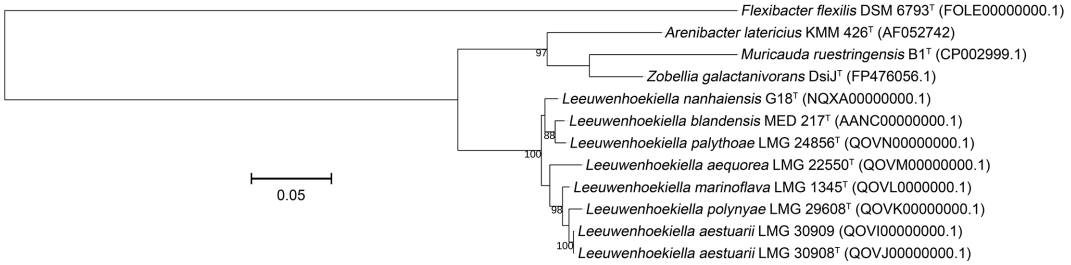

Fig. 1. Maximum-likelihood phylogenetic tree (1000 bootstraps) of complete 16S rRNA gene sequences of validly described *Leeuwenhoekiella* type strains and representative members of the family Flavobacteriaceae. Only bootstrap values higher than 80% are shown. Scale bar indicates 0.05 substitutions per site. *Flexibacter flexilis* DSM 6793^T was used as an outgroup.

method used and results should therefore always be used with caution. Consequently, here, a more comprehensive analysis for species delineation was performed using whole genome data. Analysis of a set of 107 single-copy core genes, found in the majority of bacteria [36], confirmed the placement of the new strains in the genus *Leeuwenhoekiella* (Fig. 2). Furthermore, the distance between the strains and their closest cultivated neighbour was comparable as that observed between other validly described species. Genomic data also allowed the calculation of *is*DDH and ANI. For species delineation the generally accepted cutoffs for *is*DDH and ANI are 70 and 95%, respectively [46, 47]. The *is*DDH reassociation values between strains LMG 30908^T and LMG 30909, and the type strains of the species of *Leeuwenhoekiella* were between 18.7 and 56.6% (Table 3). For ANI, these were between 75.9 and 94.4%. These data are below the recommended thresholds indicating that the isolates constitute a novel species of the genus *Leeuwenhoekiella*.

General genome characteristics of *Leeuwenhoekiella* species

Characteristics of the sequenced *Leeuwenhoekiella* genomes are listed in Table 1. Coverage was 100× for all genomes. The final assemblies and their quality were in agreement with the minimal standards for the use of genome data as proposed by Chun *et al.* [48]. The use of bcgTree for a genome-based phylogenetic analysis showed that all *Leeuwenhoekiella* genomes, except *L. aequorea* LMG 22550^T (106 of 107 genes), contained all of the 107 single-copy core genes included in the bcgTree data set [36]. Therefore it can be assumed that the genome sequences are near-complete. Genome sizes (Table 1) were similar (4.24–4.79 Mb). The genomes of *L. aequorea* LMG 22550^T and *L. palythoae* LMG 24856^T were somewhat smaller, with sizes of 3.46 and 3.94 Mb, respectively. Possibly, the reduced genome size of the latter two species may be explained by their isolation source. The type strain of *L. palythoae* was isolated from soft coral [4]. That of *L. aequorea* originates from Antarctic seawater, although a second strain was isolated from the sea urchin *Strongylocentrotus intermedius* [1]. It is well known that bacterial symbionts often have a reduced genome size and G+C content compared to free-living bacteria [49]. Although the difference is relatively small, the reduced genome sizes of *L. aequorea* LMG 22550^T and *L. palythoae* LMG 24856^T may reflect a host-associated or symbiotic life style.

Based on the genome analysis, the G+C contents of strains LMG 30908^T and LMG 30909 were 37.80 and 37.83 mol%, respectively, which are within the range known for the genus *Leeuwenhoekiella* (35–43 mol%) (Table 1) [3]. G+C content as determined here by using whole genome sequences showed small differences with the G+C content as previously

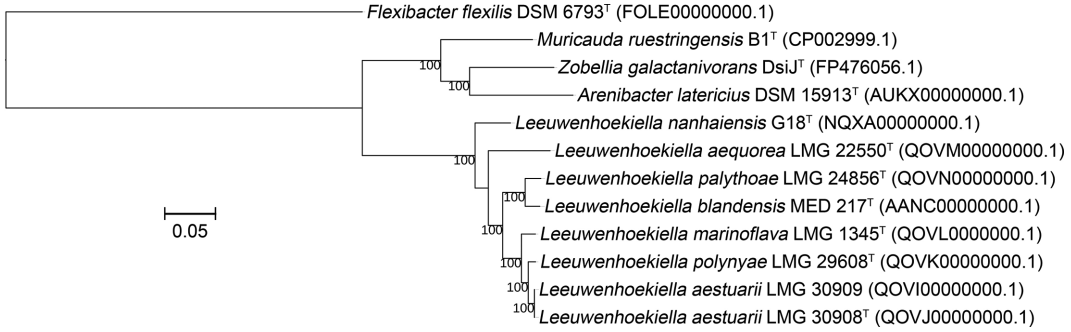

Fig. 2. Maximum-likelihood phylogenetic tree (1000 bootstraps) built using the concatenated sequence of 107 highly conserved single copy genes extracted from reference *Leeuwenhoekiella* genomes and representative members of the family *Flavobacteriaceae*. Only bootstrap values higher than 80% are shown. *Flexibacter flexilis* DSM 6793^T was used as an outgroup. Bar, 0.05 substitutions per site.

Table 3. Results (%) of average nucleotide identity (ANI) and *in silico* DNA–DNA hybridization (*is*DDH) analyses between representative genomes of *Leeuwenhoekiella* species

Strains: 1, LMG 30908^T; 2, LMG 30909; 3, *Leeuwenhoekiella marinoflava* LMG 1345^T; 4, *Leeuwenhoekiella aequorea* LMG 22550^T; 5, *Leeuwenhoekiella palythoae* LMG24856^T; 6, *Leeuwenhoekiella blandensis* MED 217^T; 7, *Leeuwenhoekiell apolynyae* LMG29608^T; 8, *Leeuwenhoekiella nanhaiensis* G18^T. Values in the lower (in bold) and upper triangle correspond to ANI and *is*DDH, respectively

Strain	1	2	3	4	5	6	7	8
1		98.7	30.9	18.7	20.4	20.6	56.6	19.0
2	**99.8**		30.9	18.8	20.3	20.6	56.8	19.0
3	**85.8**	**85.9**		20.5	20.6	20.7	32.8	19.4
4	**75.9**	**75.9**	**76.7**		18.2	18.8	19.9	19.0
5	**77.6**	**77.6**	**77.7**	**75.0**		26.4	20.3	19.7
6	**77.7**	**77.7**	**78.1**	**75.1**	**83.3**		20.8	19.6
7	**94.4**	**94.4**	**86.8**	**76.6**	**77.7**	**77.8**		19.6
8	**75.6**	**76.1**	**75.9**	**74.4**	**75.7**	**75.6**	**76.4**	

determined by the thermal denaturation method in the *Leeuwenhoekiella* species descriptions [50]. However, very large differences could be observed for *L. blandensis* MED 217^T and *L. nanhaiensis* G18^T. For these two strains, a difference of 2.75 and 7.10 mol% could be observed, respectively.

All currently known *Leeuwenhoekiella* species were isolated from seawater environments. In such ecosystems, it is estimated that there are ~10 phages in existence for each bacterial cell [51, 52]. To see whether there might be frequent contact with phages, all genomes were screened for the presence of CRISPR regions which are thought to encode functions for the prevention of infections with alien DNA [53]. Using CRISPRCasFinder [26], most genomes were found to contain one or more of these regions, except for *L. aestuarii* LMG 30908^T and *L. marinoflava* LMG 1345^T (Table 1). Although the majority of these regions was marked as questionable, the genomes of *L. palythoae* LMG 24856^T, *L. blandensis* MED 217^T and *L. polynyae* LMG 29608^T all contained one region that was marked with high evidence as a CRISPR. This was confirmed by the presence of multiple *cas* genes in the annotated genomes of the latter three strains. The absence of *cas* genes in the other genomes may indicate that the regions marked as questionable in these genomes are not CRISPRs. Additionally, the genomes were screened for the presence of prophage sequences. Interestingly, only the latter three strains were found to contain one or more of these regions. However, they were all marked as putative and incomplete (Table 1).

Leeuwenhoekiella species core metabolism

The genomes of the type strains of the genus *Leeuwenhoekiella* allow a first insight in the (core) metabolism of these organisms. Using OrthoFinder and the PATRIC server, the core genome of *Leeuwenhoekiella* consisted of 2163 proteins.

Based on the annotated genome sequences, the central metabolism of *Leeuwenhoekiella* species very likely involves the glycolysis, tricarboxylic acid cycle and the pentose phosphate pathway. By using either α- or β-D-glucose, glycolysis can be started. This was also corroborated by the API and Biolog assays. Upon completion, the resulting pyruvate may be converted into acetyl CoA. This way, glycolysis is linked to the tricarboxylic acid cycle. Additionally, the presence of all key enzymes of the pentose phosphate pathway in the annotated genomes suggests that this pathway can supply building blocks and NADPH which may be used for other biosynthetic processes. Key enzymes for photosynthesis and carbon fixation are missing in all genomes.

Interestingly, all type strains, except *L. aequorea* LMG 22550^T, were able to use arabinose in API 50 CH tests (Table S2). Analysis of the genomes confirmed this property as all genomes, with the exception of the *L. aequorea* LMG 22550^T genome, contained a conserved region that included genes involved in the arabinose metabolism. Each region contained three structural genes of the arabinose operon (i.e. *araA*, *araB*, *araD*), responsible for the conversion of arabinose into D-xylulose 5-phosphate. Additionally, the genes *galM* which encodes an epimerase capable of interconverting L- and D-arabinose, and *yidK* encoding a Na^+/solute symporter were present. Therefore, the presence of this region indicates the organisms should be able to use this sugar by channelling it to their pentose phosphate pathway. Arabinose is one of several carbohydrate components of extracellular polymeric substances produced by diatoms and other microalgae [54] and thus may be a nutrient for diatom associated strains of *L. aestuarii*.

In marine habitats, (bio)polymers/polysaccharides, produced by organisms such as phytoplankton, are omnipresent in the water column and settle and accumulate in the sediment. Contrary to terrestrial polysaccharides, marine polymers are highly sulfated. Micro-organisms play important roles in the degradation and utilization of these compounds by hydrolysing them to smaller substrates through combined action of various peptidases and proteases, and by the secretion of extracellular enzymes [16, 55–57]. To perform these tasks, a unique feature of members of the Bacteroidetes is the presence

of polysaccharide utilization loci (PULs) in their genomes [58]. These PULs encode a complement of cell surface glycan-binding proteins, TonB-dependent receptors, Carbohydrate-active enzymes and carbohydrate sensors or transcriptional regulators [59]. In members of the Bacteroidetes, these PULs constitute the major nutrient acquisition strategy [59]. PULs are characterized by the presence of at least one sequential pair of *susC* and *susD* genes involved in polysaccharide uptake in the class Flavobacteriia [60]. All *Leeuwenhoekiella* genomes analysed here contained a large number of these *susC*/*susD* pairs. The carbohydrate-active enzymes found in PULs are often glycoside hydrolases (GHs), polysaccharide lyases (PLs) and carbohydrate esterases (CEs). All *Leeuwenhoekiella* genomes analysed contained a large number of GHs per Mb (i.e. 20 to 25, Table 1), many of which were located in PULs. Additionally, the number of GHs per Mb present in *Leeuwenhoekiella* genomes is higher compared to other marine bacteria. *L. aequorea* LMG 22550^T was an exception with only 13 GHs per Mb, distributed over fewer GH families compared to other *Leewenhoekiella* species (Table 1). A similar observation could be made for CEs (Table 1).

PULs also often contain peptidases and sulfatases. All genomes contained between 33 and 40 peptidases per Mb (Table 1), which is similar to that of other marine Flavobacteria, but significantly more than other marine bacteria and also than the average bacterium [61]. The genomic peptidase composition was similar for all genomes, with approximately half of the peptidases belonging to the S9, S16, S41, M1, M16, M20, M22, M23, M24 and M28 families. The number of sulfatases in the genomes ranged between 1 and 15 (Table 1). *Leeuwenhoekiella aequorea* LMG 22550^T was again an exception with only one sulfatase present. Interestingly, the genome of *L. nanhaiensis* G18^T contained 15 sulfatases, which is three times higher than the number of sulfatases present in the other genomes (Table 1). Most of the sulfatases present were annotated as arylsulfatase and arylsulfatase A-like. These cleave sulfate esters to supply the organism with sulfur [62]. Given that most marine polysaccharides are highly sulfated [57], these sulfatases may aid *Leeuwenhoekiella* species to degrade sulfated polysaccharides and subsequently use them as a food source.

The presence of large numbers of peptidases, GHs and CEs was confirmed by API and Biolog tests which showed all strains utilized various labile carbon sources and showed extracellular activities associates with protein, polysaccharide and lipid hydrolysis (Tables S1– 4). These observations hint at a dedicated role of *Leeuwenhoekiella* species as (bio)polymer degraders, with a specialization on degrading proteins. This was also confirmed by a higher diversity of peptidases than GHs. Additionally, specialization in degradation of proteins and other high molecular weight compounds often results in a lowered capacity for usage of low molecular weight compound usage as is reflected in a lower number of transporters in the genome relative to other bacteria (Table 1) [61].

The presence of nitronate monooxygenase in all annotated genomic sequences suggests that the conversion of nitroalkanes – often found in the environment as a result of human activities [63] – into nitrite may be important for the nitrogen metabolism. The presence of a nitrate/nitrite transporter and nitrite reductase in the genomes of strains LMG 30908^T and LMG 30909, and the type strains of *L. marinoflava*, *L. aequorea* and *L. nanhaiensis* suggests that these organisms can potentially also rely on the uptake and/or conversion of extracellular nitrate and nitrite. Additionally, the presence of NarB and NirB/D in the annotated genomes of *L. marinoflava* LMG 1345^T, *L. palythoae* LMG 24856^T, *L. nanhaiensis* G18^T and *L. aestuarii* LMG 30908^T and LMG 30909 suggests these organisms may also be capable of converting nitrate to nitrite and nitrite to ammonia, respectively.

Phosphate is one of the essential nutrients for bacteria. All currently described *Leeuwenhoekiella* species originate from marine environments, where phosphate concentrations usually are very low and can substantially differ between sites and over time [64, 65]. To overcome these variable conditions of the environment and cope with the scarcity of this nutrient, *Leeuwenhoekiella* species may rely on a phosphate regulon [66, 67]. This regulon plays a key role in phosphate homeostasis. As observed from the annotated *Leeuwenhoekiella* genomes, the phosphate regulon is controlled by a two-component regulatory system comprised of an inner-membrane histidine kinase sensor (PhoR) and a cytoplasmatic transcriptional response regulator (PhoP). During phosphate deprivation in *Leeuwenhoekiella* species, PhoR-PhoP is required for transcription of several other important genes of the phosphate regulon (e.g. the phosphate scavengers PhoA, PhoD and GlpQ). This way, *Leeuwenhoekiella* species may act against phosphate starvation [66, 68].

All but one of the *Leeuwenhoekiella* strains tested grew under anaerobic conditions (Table 4), although no clear explanation for this could be found in the annotated genomes. In marine systems, O_2 concentrations may significantly vary due to the presence of oxygen minimum zones and can go as low as 1 nM l^{-1} [69]. Zakem and Follows [69] showed that the critical limit for oxygen intake of aerobic marine bacteria was approximately 1–10 nM l^{-1}. Below this limit, microbes either die or switch to anaerobic forms of respiration. This limit may thus explain growth of *Leeuwenhoekiella* species under anaerobic conditions in a laboratory environment (Table 4), as anaerobic cabinets very often contain small traces of oxygen (~10–50 p.p.m.). These traces are at least 100-fold higher than the critical limit and may thus be enough for the strains to grow. In the absence of indication for anaerobic growth in the genomes, we hypothesize that the *Leeuwenhoekiella* strains can grow aerobically at relatively low (<50 p.p.m.) oxygen concentrations which may be relevant in low oxygen marine habitats. Only *L. nanhaiensis* LMG30890^T (Table 4) did not grow in the anaerobic cabinet. This may indicate that this organism needs more oxygen to grow.

Given the marine nature of the strains, swimming or gliding motility and adhesion to surfaces may give them a competitive advantage in the environment, because of the constant competition for space and nutrients [70]. Therefore, the

Table 4. Phenotypic characteristics of strains LMG 30908^T, LMG 30909, R-46770, R-50233 and type strains of related species of the genus *Leeuwenhoekiella*

Strains: 1, LMG 30908^T; 2, LMG 30909; 3, R-46770; 4, R-50233; 5, *Leeuwenhoekiella marinoflava* LMG 1345^T; 6, *Leeuwenhoekiella aequorea* LMG 22550^T; 7, *Leeuwenhoekiella palythoae* LMG24856^T; 8, *Leeuwenhoekiella blandensis* MED 217^T; 9, *Leeuwenhoekiella polynyae* LMG29608^T; 10, *Leeuwenhoekiella nanhaiensis* LMG30890^T. Results of API 20 NE, API 50 CH, API ZYM and Biolog GEN III assays can be found in Tables S1–S4. All strains formed yellow/orange colonies, have rod-shaped cell morphology and are positive for gliding motility, catalase, oxidase, growth under microaerobic conditions and hydrolysis of Tweens 20, 40, 60 and 80. All strains are negative for Gram stain, formation of spores and hydrolysis of CM cellulose. All strains are sensitive to vancomycin (30 µg) and chloramphenicol (30 µg), but resistant to gentamicin (10 µg). +, positive; –, negative; R, resistant; S, sensitive.

Characteristic	1	2	3	4	5	6	7	8	9	10
Cell size (µm)	2.0–3.0×0.4–0.6	2.5–3.0×0.4–0.6	3.0–3.5×0.5	3.0–4.0×0.5	2.0–3.0×0.4–0.6	2.0–3.0×0.4–0.6	2.0–3.0×0.4–0.5	3.0–4.0×0.4–0.6	2.0–3.0×0.4–0.5	1.4–4.1×0.4–0.7
Anaerobic	+	+	+	+	+	+	+	+	+	–
Growth conditions:										
Temperature range (°C)	4–41	10–37	4–41	4–41	4–37	4–37	4–44	4–37	10–41	4–41
Temperature optimum (°C)	20–30	20–30	20–30	20–30	20–30	20–30	20–30	20–30	25–30	25–30
Salinity range (% NaCl, w/v)	0.5–12	0.5–12	0.5–12	0.5–12	0.5–15	0.5–15	0.5–15	0–15	0.5–15	0.5–12
Salinity optimum (% NaCl, w/v)	2–7	2–5	2–6	2–5	1–8	2–5	1–4	0.5–5	1–8	2–5
pH range	5.5–9.0	5.5–9.0	5.5–9.0	5.5–9.0	5.5–9.0	5.5–9.0	5.5–9.0	5.0–9.0	5.5–8.5	5.5–8.5
pH optimum	6.0–6.5	6.0–6.5	6.0–6.5	6.5	6.0	6.0	6.0	6.0–6.5	6.0–7.0	6.5–7.5
Susceptibility to:										
Ampicillin	S	S	S	S	S	S	R	S	R	S
Tetracycline	R	R	R	R	S	R	R	R	R	R
Bacitracin	S	S	S	S	S	R	S	S	S	S
Hydrolysis of:										
Casein	+	+	+	+	+	+	–	–	+	–
Starch	–	–	–	–	+	+	+	+	+	+
DNA	+	+	+	+	–	–	–	–	–	–

genomes were screened for the presence of genes involved in these processes. Using the Pfam search included in the annotation using the IMG-ER platform, all genomes were found to contain between 12 and 18 domains involved in surface adhesion (Table S5), which represented between three and five adhesion genes per Mb. These numbers are in line with previous results for other marine Bacteroidetes, but higher compared to marine Proteobacteria [61]. Additionally, all genomes contained the set of genes necessary for gliding motility (i.e. *gldA-gldN*) [71], a characteristic which was confirmed by laboratory testing (Table 4). Another important feature for adhesion may be the presence of glycosyl transferases in the genomes. These proteins, often positioned in the outer membrane, generate polysaccharides for several functions, including adhesion. All *Leeuwenhoekiella* genomes contained between 27 and 32 of these glycosyl transferases. Taken together, the high presence of the aforementioned three features in all genomes indicate that gliding motility and adhesion to surfaces is important for *Leeuwenhoekiella* species

CHARACTERISTICS OF *LEEUWENHOEKIELLA AESTUARII* SP. NOV.

The whole-cell fatty acid composition of the four new strains was similar to those of the other validly described species (Table 5).

The yellow-orange pigment of all strains could be extracted using pure methanol. After spectrometric analysis, the pigments of the four different strains all gave absorption spectra with absorption maxima at ~335 and ~449 nm, and a small shoulder peak at approximately 422 nm. Alkalization with 0.1 M NaOH did not lead to a shift in the peak positions, indicating that none of the strains produce the flexirubin pigment. This was confirmed by flooding the extracts with a 20% KOH solution which did not change the colour of the extract from yellow-orange to brown. Based on the absorption maxima, pigment colour and data from the annotated genomes, the main pigment is likely 7,8-dihydro-β-carotene.

Strains of the newly proposed species originate from estuarine intertidal sediments or from diatom cultures originating from the same sediments. The four new strains grew in a broad salinity range of 0.5–12% (w/v NaCl) which may be an adaptation to the variable salinity levels in estuarine systems. Furthermore, growth of strains LMG 30908^T, R-46770 and R-50233 was observed in the rather wide temperature range of 4–41 °C, whereas for strain LMG 30909^T this was 10–37 °C. This may also reflect adaptation to the local conditions. Indeed, the intertidal sediment flats at Paulina salt marsh comprise many shallow puddles with diatom biofilms that may intermittently become warmer under exposed sunny conditions (personal observation). Since the type strains of the other species of *Leeuwenhoekiella* were also able to grow in similar ranges of salinity and temperature (Table 4), these features may be genus-wide adaptations.

A comparison of the morphological and physiological characteristics of strain LMG 30908^T and other validly described type strains of the genus *Leeuwenhoekiella* are listed in Table 4. A complete overview of all chemotaxonomical results as obtained by performing API 20NE, API 50CH, API ZYM and the Biolog GEN III MicroPlate tests can be found in Tables (S1-s4). As shown in (Tables 4 and S1–S4, strain LMG 30908^T shared many characteristics with other species of the genus *Leeuwenhoekiella*, for example the ability to hydrolyse Tween 20, 40, 60 and 80, but not CM cellulose. However, it also showed several differences, for example the presence of α-galactosidase activity and the ability to hydrolyse starch, but not DNA (Table 4). Thus, based on phylogenetic, phenotypic and chemotaxonomic characteristics, the four strains LMG 30908^T, LMG 30909, R-46770 and R-50233 are considered to represent a novel species of the genus *Leeuwenhoekiella*, for which the name *Leeuwenhoekiella aestuarii* is proposed.

DESCRIPTION OF *LEEUWENHOEKIELLA AESTUARII* SP. NOV.

Leeuwenhoekiella aestuarii (a.es.tu.a.ri'i. L. gen. n. *aestuarii*, of a tidal flat from an estuary, from where the type strain was isolated).

Cells are Gram-negative, oxidase- and catalase-positive, rod-shaped (0.4–0.6×2.0–4.0 µm) and motile by gliding. When grown on MA, colonies are yellow/orange, circular and 0.5–2 mm in diameter. Growth is observed at 4–41 °C with an optimum of 20–30 °C, and at pH 5.5–9.0 with an optimum of 6.0–6.5. Grows with 0.5–12% (w/v) NaCl (optimum, 2–5%, w/v). Growth occurs under aerobic atmosphere, but also under microaerobic (80% N_2, 15% CO_2 and 5% O_2) and anaerobic atmospheres (80% N_2, 10% CO_2 and 10% H_2, <50 p.p.m. O_2). Does not reduce nitrate. Hydrolysis of DNA, Tweens 20, 40, 60 and 80, and casein are positive. Does not hydrolyse starch and carboxymethylcellulose. Does not produce flexirubin.

In the API ZYM gallery, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cysteine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase and *N*-acetyl-β-glucosaminidase activities are present. Lipase (C14), α-chymotrypsin, β-glucuronidase, α-mannosidase and α-fucosidase activities are absent.

According to the API 20 NE gallery, strain LMG 30908^T is positive for arginine dehydrolase, urease, aesculin, the PNPG test (β-galactosidase) and assimilation of glucose, arabinose, mannose, mannitol, *N*-acetyl-glucosamine, maltose, gluconate, adipic acid, malate, citrate and phenylacetic acid.

In the API 50 CH assay, positive results are obtained for L-arabinose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, amygladin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, melizitose, raffinose and turanose.

In Biolog GEN III assays, positive reactions are obtained for the following carbon sources: dextrin, maltose, trehalose,

Table 5. Fatty acid composition of strains lmg 30908^T, lmg 30909, R-46770, R-50233 and type strains of related species of the genus *Leeuwenhoekiella*

Strains: 1, LMG 30908^T; 2, LMG 30909; 3, R-46770; 4, R-50233; 5, *Leeuwenhoekiella marinoflava* LMG 1345^T; 6, *Leeuwenhoekiella aequorea* LMG 22550^T; 7, *Leeuwenhoekiella palythoae* LMG 24856^T; 8, *Leeuwenhoekiella blandensis* LMG 28563^T; 9, *Leeuwenhoekiella polynyae* LMG 29608^T; 10, *Leeuwenhoekiella nanhaiensis* G18^T. TR, Trace amount (i.e. <1%). –, Not detected. Values shown are percentages of total fatty acids obtained in this study.

Fatty acid		1	2	3	4	5	6	7	8	9	10
$C_{12:00}$		TR	TR	TR	TR	1.0	1.0	TR	1.0	TR	TR
$C_{15:00}$		3.7	3.7	4.3	3.8	7.0	5.7	2.8	4.9	4.7	5.7
$C_{15:0}$ 2-OH		TR	1.0	1.1	1.0	1.5	1.5	TR	1.0	1.0	1.0
$C_{15:0}$ 3-OH		–	–	–	–	1.0	–	–	–	–	–
anteiso-$C_{15:0}$		3.3	3.1	3.5	3.2	4.1	6.8	2.0	2.9	3.1	3.9
iso-$C_{15:0}$		15.4	14.5	13.1	15.8	16.6	17.9	14.7	16.4	14.7	15.4
iso-$C_{15:0}$ 3-OH		2.4	2.8	2.5	2.8	3.3	2.0	3.2	3.0	2.7	2.5
anteiso-$C_{15:1}$ A		TR	TR	1.4	TR	TR	TR	TR	TR	TR	TR
iso-$C_{15:1}$ G		14.7	14.4	16.6	13.9	11.2	7.3	15.8	10.0	11.3	11.2
$C_{16:00}$		1.0	TR	1.0	TR	1.3	TR	TR	TR	1.3	TR
$C_{16:0}$ 3-OH		TR	TR	1.0	TR	–	–	–	–	TR	TR
iso-$C_{16:0}$		1.1	1.1	1.8	1.3	1.2	2.7	TR	1.8	1.0	1.4
iso-$C_{16:0}$ 3-OH		1.5	1.8	2.6	1.6	1.7	3.0	1.7	2.1	1.7	2.2
$C_{17:0}$ 2-OH		3.1	3.7	3.7	3.5	4.5	6.1	1.9	3.1	3.2	4.9
iso-$C_{17:0}$		1.0	1.2	TR	1.3	TR	TR	TR	TR	1.0	TR
iso-$C_{17:0}$ 3-OH		13.6	17.5	14.0	16.4	18.1	12.9	19.3	19.5	15.0	18.1
$C_{17:1}\omega6c$		TR	TR	TR	TR	1.0	1.1	TR	1.6	1.0	1.2
iso-$C_{19:0}$		1.0	1.4	TR	1.3	TR	TR	1.7	TR	1.0	1.2
anteiso-$C_{17:1}\omega9c$		–	–	–	–	–	1.6	–	–	–	–
iso-$C_{17:1}\omega9c$		8.6	8.2	7.5	8.6	7.1	10.5	9.6	9.7	10.2	9.1
Summed features:*											
3	$C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH	19.6	17.0	16.2	16.6	12.0	11.4	17.1	14.4	18.6	10.1
4	iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B	1.4	1.5	1.6	1.6	–	TR	1.1	1.0	1.1	2.3
Unknown ECL 13.565†		2.0	1.6	1.1	1.1	1.7	1.2	1.6	1.6	2.5	1.9

*Summed features represent groups of multiple fatty acids that cannot be separated by the Microbial Identification System.
†Unknown fatty acid with chain length (ECL) 13.565.

cellobiose, gentiobiose, sucrose, turanose, stachyose, raffinose, lactose, melibiose, methyl β-D-glucoside, D-salicin, *N*-acetyl-D-glucosamine, α-D-glucose, D-mannose, D-fructose, D-galactose, L-fucose, L-rhamnose, glycerol, D-glucose-6-PO_4, D-fructose-6-PO_4, gelatin, glycyl-L-proline, L-arginine, L-aspartic acid, L-glutamic acid, L-serine, pectin, D-galacturonic acid, L-galactonic acid lactone, D-gluconic acid, D-glucuronic acid, methyl pyruvate, L-lactic acid, D-malic acid, L-malic acid, bromo-succinic acid, Tween 40, acetoacetic acid, acetic acid and formic acid. For the chemical sensitivity assays, positive reactions are obtained for pH 6, pH 5, 1% NaCl, 4% NaCl, 8% NaCl, 1% sodium lactate, D-serine, troleandomycin, rifamycin SV, minocycline, lincomycin, vancomycin, nalidixic acid, lithium chloride, potassium tellurite, aztreonam and sodium butyrate.

Susceptible to vancomycin, tetracycline and chloramphenicol, but resistant to ampicillin, bacitracin and gentamicin.

The major cellular fatty acids are co-eluted fatty acids $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH, and fatty acids iso-$C_{15:0}$, iso-$C_{15:1}$ G and iso-$C_{17:0}$ 3-OH.

The DNA G+C content of the type strain as derived from the genome sequence is 37.80mol%. The approximate genome

size is 4.43 Mb. The DNA G+C content of strain R-50232, as derived from the genome sequence is 37.83%, its approximate genome size 4.36 Mb. GenBank deposits of strains R-48165^T and R-50232 are SAMN09487585 and SAMN09487586, respectively. The IMG-ER genome IDs of strains R-48165^T and R-50232 are 2772190913 and 2772190914, respectively

The type strain, R-48165^T (=LMG 30908^T=CECT 9775^T=DSM 107866^T), as well as strains R-50232 (=LMG 30909), R-46770 and R-50233 were isolated from intertidal sediment from the Paulina salt marsh, The Netherlands.

EMENDED DESCRIPTION OF THE GENUS *LEEUWENHOEKIELLA* NEDASHKOVSKAYA *ET AL.* 2005 EMEND. NEDASHKOVSKAYA *ET AL.* 2014

The description is as given by Nedashkovskaya *et al.* [1] and [2] with the following additions. The DNA G+C content from the draft genomes of the type strains is between 35.4 and 42.1 mol%, the approximate genome size is between 3.4 and 4.8 Mb.

EMENDED DESCRIPTION OF *LEEUWENHOEKIELLA MARINOFLAVA* NEDASHKOVSKAYA *ET AL.* 2005 EMEND NEDASHKOVSKAYA *ET AL.* 2014

The description of *L. marinoflava* is as given by Nedashkovskaya *et al.* [1] and [2] with the following additions. The DNA G+C content as derived from the draft genome sequence of the type strain LMG 1345^T is 37.52%, its approximate size is 4.74 Mb, its GenBank deposit SAMN09487581, its IMG-ER genome ID 2772190910. The type strain grows under aerobic atmosphere, but also under microaerobic (80% N_2, 15% CO_2 and 5% O_2) and anaerobic atmosphere (80% N_2, 10% CO_2 and 10% H_2, <50 p.p.m. O_2). Additional features are listed in Tables 4 and S1–S4.

EMENDED DESCRIPTION OF *LEEUWENHOEKIELLA AEQUOREA* NEDASHKOVSKAYA *ET AL.* 2005 EMEND. NEDASHKOVSKAYA *ET AL.* 2014

The description of *L. aequorea* is as given by Nedashkovskaya *et al.* 2005 [1] and Nedashkovskaya *et al.* 2014 [2] with the following additions. The DNA G+C content as derived from the draft genome sequence of the type strain LMG 22550^T is 35.41 mol%, its approximate size is 3.46 Mb, its GenBank deposit SAMN09487582, its IMG-ER genome ID 2772190911. Growth is observed at pH 5.5–9.0 (optimum, pH 6.0). Growth occurs under aerobic atmosphere, but also under microaerobic (80% N_2, 15% CO_2 and 5% O_2) and anaerobic atmosphere (80% N_2, 10% CO_2 and 10% H_2, <50 p.p.m. O_2). Additional features are listed in Tables 4 and S1–S4.

EMENDED DESCRIPTION OF *LEEUWENHOEKIELLA PALYTHOAE* NEDASHKOVSKAYA *ET AL.* 2009

The description of *L. palythoae* is as given by Nedashkovskaya *et al.* 2009 [4] with the following additions. The DNA G+C content as derived from the draft genome sequence of the type strain LMG 24856^T is 39.96 mol%, its approximate size is 3.94 Mb, its GenBank deposit SAMN09487583, its IMG-ER genome ID 2772190912. Growth is observed at pH 5.5–9.0 (optimum, 6.0). Growth occurs under aerobic atmosphere, but also under microaerobic (80% N_2, 15% CO_2 and 5% O_2) and anaerobic atmosphere (80% N_2, 10% CO_2 and 10% H_2, <50 p.p.m. O_2). Additional features are listed in Tables 4 and S1–S4.

EMENDED DESCRIPTION OF *LEEUWENHOEKIELLA BLANDENSIS* PINHASSI *ET AL.* 2006

The description of *L. blandensis* is as given by Pinhassi *et al.* 2006 [5] with the following additions. The DNA G+C content as derived from the draft genome sequence of the type strain MED 217^T is 39.76 mol%, its approximate size is 4.24 Mb, its GenBank deposit SAMN02436111, its IMG-ER genome ID 638341115. Growth is observed at pH 5.0–9.0 (optimum, pH 6.0–6.5). Growth occurs under aerobic atmosphere, but also under microaerobic (80% N_2, 15% CO_2 and 5% O_2) and anaerobic atmospheres (80% N_2, 10% CO_2 and 10% H_2, <50 p.p.m. O_2). Additional features are listed in Tables 4 and S1–S4.

EMENDED DESCRIPTION OF *LEEUWENHOEKIELLA POLYNYAE* SI *ET AL.* 2015

The description of *L. polynyae* is as given by Si *et al.* 2015 [6] with the following additions. The DNA G+C content as derived from the draft genome sequence of the type strain LMG 29608^T is 38.11 mol%, its approximate size is 4.79 Mb, its GenBank deposit SAMN09487584, its IMG-ER genome ID 2751185537. Growth occurs under aerobic atmosphere, but also under microaerobic (80% N_2, 15% CO_2 and 5% O_2) and anaerobic atmospheres (80% N_2, 10% CO_2 and 10% H_2, <50 p.p.m. O_2). Additional features are listed in Tables 4 and S1–S4.

EMENDED DESCRIPTION OF *LEEUWENHOEKIELLA NANHAIENSIS* LIU *ET AL.* 2016

The description of *L. nanhaiesnsi* is as given by Liu *et al.* 2016 [3] with the following additions. The DNA G+C content as derived from the draft genome sequence of the type strain G18^T is 42.10 mol%, its approximate size is 4.36 Mb, its GenBank deposit SAMN07515617, its IMG-ER genome ID 2775506714. Growth occurs under aerobic atmosphere,

but also under microaerobic (80% N_2, 15% CO_2 and 5% O_2) but not under anaerobic atmosphere (80% N_2, 10% CO_2 and 10% H_2, <50 p.p.m. O_2). Additional features are listed in Tables 4 and S1–S4

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

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