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Proposal of *Henriciella barbarensis* sp. nov. and *Henriciella algicola* sp. nov., stalked species of the genus and emendation of the genus *Henriciella*

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Abstract

Two Gram-negative, heterotrophic, aerobic, prosthecated, marine bacteria, designated strains MCS23^T and MCS27^T, were isolated from seawater samples. NaCl was required for growth. The major polar lipid detected in strain MCS27^T was phosphatidylglycerol, whereas those detected in MCS23^T were phosphatidylglycerol, sulfoquinovosyl diacylglycerol and 1,2-diacyl-3-α-D-glucuronopyranosyl-sn-glycerol taurineamide. The most abundant cellular fatty acids were $C_{18:1}\omega7$ and $C_{16:0}$, hydroxyl-fatty acids were 3-OH $C_{12:0}$ in both strains and 3-OH $C_{11:0}$ in MCS23^T. Strains MCS23^T and MCS27^T had DNA G+C contents of 57.0 and 55.0 mol%, respectively. The two strains shared 99.3 % 16S rRNA gene sequence similarity; levels of similarity with the type strains of species of the genus *Henriciella* were 99.4–97.8 % but DNA–DNA hybridizations were 53 % or lower. Besides their 16S rRNA gene sequences, the novel strains can be differentiated from other species of the genus *Henriciella* by cell morphology, lipid and fatty acid patterns and enzyme activities. The data obtained led to the identification of two novel species, for which the names *Henriciella barbarensis* sp. nov. (type strain MCS23^T=LMG 28705^T=CCUG 66934^T) and *Henriciella algicola* sp. nov. (type strain MCS27^T=LMG 29152^T=CCUG 67844^T) are proposed. As these two novel species are the first prosthecate species in the genus *Henriciella*, an emended genus description is also provided.

Cauloform bacteria are dimorphic, prosthecate bacteria, which reproduce by separation of two cells that are morphologically different from each other. One of these cells is non-motile and sessile by means of a prosthecum, and the other cell is flagellated and motile, bearing one polar flagellum [1]. The mode of reproduction of the dimorphic prosthecate bacteria reflects their life in oligotrophic habitats by fostering the dispersion of the population at each generation, minimizing competition for resources [2]. It took more than 100 years after the report of the first isolation of a *Caulobacter* sp. [3] to realize, by analysing the 16S rRNA genes, that these bacteria belonged to two different families, *Caulobacteraceae* and *Hyphomonadaceae* [4, 5]. A polyphasic study of a large set of cauloform isolates revealed that these strains belonged to the genera *Caulobacter*, *Brevundimonas*, *Phenylobacterium* and *Maricaulis* [6, 7]. Some isolates, however, were so different from all known species that they could not be assigned to one of them. We report here the characterization of two marine isolates obtained from different habitats that have their closest relatives in the genus *Henriciella* but are prosthecated and sufficiently distinct from its accepted members that we propose they represent two novel species of the genus *Henriciella*.

The strains of this study were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ); the Laboratory of Microbiology, Department of Biochemistry and Microbiology, Faculty of Sciences of Ghent University (LMG); and from John Smit of the University of British Columbia (MCS strains). All strains were grown in the marine-*Caulobacter*-medium SPYEM: 30 g sea salts (Sigma), 0.5 g NH_4Cl, 1 l deionized water. After autoclaving and cooling, 20 ml 50xPYE, 2 ml 50 % glucose (sterile) and 5 ml riboflavin (0.2 mg ml^{-1}, sterile filtrated) were added. 50xPYE: 10 % peptone and 5 % yeast extract in deionized water (autoclaved). The strains were cultivated in 2-l flasks at 30 °C, shaken at 100 r.p.m. and the biomass was harvested in the late exponential phase after 72 h. Anaerobic growth

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Abbreviation: FAB, fast atom bombardment.
The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains MCS23^T and MCS27^T are AJ227807 and KP722025, respectively.
Three supplementary tables and a supplementary figure is available with the online Supplementary Material.

was tested on SPYEM plates in an anaerobic chamber (GasPak; BBL Microbiology Systems) [8].

Genomic DNA was isolated from two inoculating loops full of bacterial cells using the DNeasy Blood and Tissue kit for purification of total DNA (Qiagen) with the addition of RNase A (Sigma), according to the manufacturers' instructions. DNA was enzymatically digested as described by Gehrke *et al.* [9] and the mean G+C content was determined by HPLC [10]. Calculations were carried out according to Mesbah *et al.* [11], with non-methylated lambda-phage DNA (Sigma) as a standard. The G+C contents of MCS23^T (57.0 mol%) and MCS27^T (55.0 mol%) were well within the range described for the genus *Henriciella* [12].

For 16S rRNA gene sequencing and analysis, single colonies were picked from plates, suspended in 100 µl TE buffer and boiled for 5 min. The suspension was briefly centrifuged and 1 µl of the supernatant was used for PCR. A nearly complete 16S rRNA gene sequence was obtained as described previously [6] and the final contig was assembled using the program SEQUENCHER version 4.0.5 (Gene Codes Corporation). The 16S rRNA gene sequences of the type strains of species of the genus *Henriciella* were obtained from the EMBL database [13]. The nucleotide sequences were aligned using the evolutionary conserved primary sequence and secondary structure [14] as references. Evolutionary distances [15] were calculated using CLUSTAL Omega software [16] using only identical, unambiguously determined nucleotide positions. Tree topologies were calculated with MEGA 6.06 software [17] applying the maximum-likelihood algorithm (Fig. 1), and the neighbour-joining algorithm with 500 bootstrap replications (Fig. S1, available in the online Supplementary Material).

From the analysis of the 16S rRNA gene sequences, species of the genus *Henriciella* were found to be the closest relatives of strains MCS23^T and MCS27^T. The similarity of the 16S rRNA gene sequence of strain MCS23^T to *Henriciella marina* DSM 19595^T, *Henriciella aquimarina* LMG 24711^T and *Henriciella litoralis* DSM 22014^T were 99.4 , 98.1 and 97.8 %, respectively. For strain MCS27^T, the similarities to these type strains were determined to be 98.9 , 98.5 and 98.3 %, respectively, while the similarity between MCS23^T and MCS27^T was 99.3 %. Therefore, to decide whether strains MCS23^T and MCS27^T belong to any of the three recognized species of the genus *Henriciella* or are distinct species, DNA–DNA hybridizations between these five strains were required.

DNA–DNA hybridization was done by the service of DSMZ and chromosomal DNA of high-molecular weight was isolated according to the method of Cashion *et al.* [18]. Degrees of DNA–DNA binding, expressed as percentages, were determined spectrophotometrically using the initial renaturation methods of De Ley *et al.* [19] and Huss *et al.* [20]. DNA–DNA hybridization between strains MCS 23^T and MCS 27^T gave 53 % relatedness, while DNA–DNA relatedness values between MCS23^T or MCS27^T and *H. marina* DSM 19595^T, *H. litoralis* DSM 22014^T or *H. aquimarina* LMG 24711^T were 50 % or lower (Table S1). As these values are below 70 % [21], this proved that strains MCS 23^T and MCS 27^T are both novel species within the genus *Henriciella*.

For whole-cell fatty acid analysis, cells were saponified [15 % (w/v) NaOH, 30 min, 100 °C], methylated to fatty acid methyl esters (methanolic HCl, 10 min, 80 °C) and extracted [hexane/methyl-tert-butyl ether (1 : 1, v/v)] as described in detail by Osterhout *et al.* [22]. Fatty acid methyl esters were analysed on a Hewlett-Packard 5890A gas chromatograph. Separation of fatty acid methyl esters was achieved with a fused-silica capillary column (25 m by 0.2 mm) with cross-linked 5 % phenyl methyl silicone (film thickness 0.33 µm; HP Ultra 2). The computer-controlled parameters were the same as those described by Osterhout. The instrument was equipped with a flame ionization detector and an autosampler (HP 7673). H_2 was serving as carrier gas. While $C_{18:1}$ $\omega7$ was the main fatty acid in *H. marina* DSM 19595^T, it was less dominant in strain MCS23^T, and in strain MCS27^T it was even less than $C_{16:0}$. The occurrence of $C_{20:1}\omega9$ in strain MCS23^T is novel for species of the genus *Henriciella*. 3-OH $C_{12:0}$ occurred in considerable amounts in strain MCS27^T but was accompanied by 3-OH $C_{11:0}$ in strain MCS23^T (Table 1). The detection of 3-OH $C_{12:0}$ in both MCS23^T and MCS27^T corroborated their placement within the genus *Henriciella* as this is the common hydroxyl-fatty acid of *Henriciella*.

Lipids were extracted using a modified Bligh–Dyer procedure [23] as described previously [24]. Isoprenoid quinones were analysed using an Agilent 6410 Triple Quadropole LS/MS as described by Ruiz-Jimenez *et al.* [25]. Polar lipids were analysed using Fast Atom Bombardment (FAB) mass spectrometry. FAB-MS in the positive and negative mode was performed on the first of two mass spectrometers of a tandem high-resolution instrument in a $E_1B_1E_2B_2$ configuration (JMS-HX/HX110A; JEOL) at 10 kV accelerating voltage with the resolution set to 1 : 1000. The JEOL FAB gun was operated at 6 kV with xenon. 3-Nitrobenzyl alcohol was used as matrix in the positive mode and a mixture of triethanolamine and tetramethylurea (Japanese matrix) in the negative mode. Positive and negative daughter ion spectra were recorded using all four sectors of the tandem mass spectrometer. High energy collision-induced dissociation (CID) took place in the third field free region. Helium served as the collision gas at a pressure sufficient to reduce the precursor ion signal to 30 % of the original value. The collision cell was operated at ground potential in the positive and negative modes. The resolution of MS2 was set to 1 : 1000. FAB-CID spectra (linked scans of MS2 at constant B/E ratio) were recorded with 300 Hz filtering on a JEOL DA 7000 data system.

The dominant quinone in strains MCS23^T and MCS27^T was ubiquinone-10 (99 %) as has been reported for all species of the family *Hyphomonadaceae*. The glycolipids of

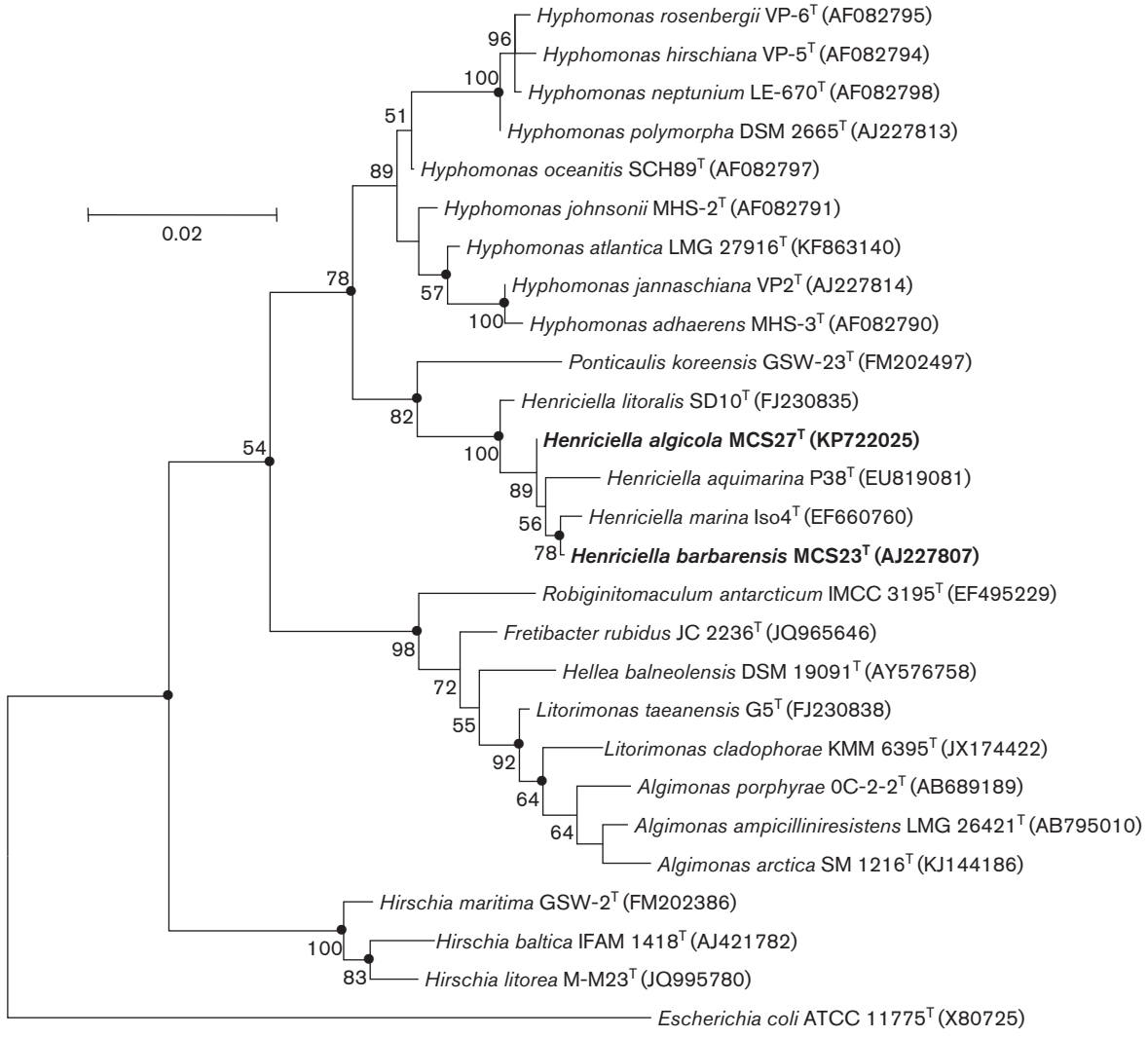

Fig. 1. Maximum-likelihood tree based on 16S rRNA gene sequences, showing the phylogenetic relationships of strains MCS23^T and MCS27^T and related taxa. Bootstrap percentages >50 % (based on 150 replications) are shown. Filled circles indicate the corresponding nodes were recovered in the maximum-parsimony and neighbour-joining trees. *Escherichia coli* ATCC 11775^{T}served as an outgroup. Bar, 0.02 substitutions per nucleotide position.

both strains MCS23^T and MCS 27^T were dominated by α-D-glucopyranosyl- and α-D-glucopyranuronosyl-diacylglycerols, also common in *Caulobacter*, *Brevundimonas*, *Maricaulis* and some other species of the *Hyphomonadaceae* (Table S2). In addition to these glycolipids, phospho- and sulfolipids could be identified. Using MS/MS the structures of several of these polar lipids could be identified as listed in Table S3. The (−)−FAB CID mass spectrum of the molecular ions showed the loss of fatty acids and the corresponding ketenes. The relative abundance of the carboxylate anions provides evidence for the relative positions of the two acyl functions where the loss at the sn-2-acyl position is favoured, thus yielding a more abundant carboxylate anion [26]. MS data led to the identification of the main phospholipids as 1-octadecenoyl-2-hexadecanoyl-*sn*-glycero-3-phosphoryI-glycerol and 1-nonadecenoyl-2-hexadecanoyl-*sn*-glycero-3-phosphoryI-glycerol, also common in the genus *Hyphomonas* but not in species of the genus *Maricaulis* [27, 28]. Sulfoquinovosyl diacylglycerol and 1,2-diacyl-3-α-D-glucuronopyranosyl-sn-glycerol taurineamide were found only in strain MCS23^T but not in MCS27^T (Table S3).

Parallel samples for routine scanning electron microscopy were fixed with 2 % glutaraldehyde and 5 % formaldehyde in cacodylate buffer, placed onto poly-L-lysine-coated 12 mm glass cover slips, fixed for 10 min with 1 % glutaraldehyde in TE buffer, dehydrated with a graded series of acetone, and critical-point-dried with liquid CO_2 (CPD 030; Bal-Tec). After sputter coating with gold-palladium (SCD

Table 1. Fatty acid content (mean percentage of total) of whole-cell hydrolysates of MCS23^T and MCS27^T and members of the genus *Henriciella*

Strains: 1, MCS23^T; 2, MCS27^T; 3, *Henriciella marina* DSM 19595^T; 4, *Henriciella aquimarina* LMG 24711^T; 5, *Henriciella litoralis* DSM 22014^T.

Fatty acid	1	2	3	4	5
3-OH $C_{11:0}$	2.1	–	–	–	–
3-OH $C_{12:0}$	1.1	8.6	1.1	4.1	–
$C_{15:0}$	3.9	4.3	2.9	1.8	2.5
$C_{16:0}$	13.4	32.8	7.8	21.0	21.2
$C_{17:0}$	19.2	10.8	4.8	4.3	10.1
$C_{17:1}\omega5$	5.7	–	11.7	3.0	7.2
$C_{17:1}\omega8$	–	3.9	–	2.7	5.5
$C_{18:0}$	1.8	3.7	–	3.4	3.3
$C_{18:1}\omega7$	29.3	30.0	53.5	38.4	41.2
11-Me-$C_{18:1}\omega5t$	–	6.0	–	8.7	3.0
$C_{20:1}\omega9$	4.8	–	–	–	0.7

500; Bal-Tec), samples were examined in a Zeiss Merlin field emission scanning electron microscope at an acceleration voltage of 5 kV with the Everhart-Thornley HE-SE-detector and Inlens SE-detector in a 25 : 75 ratio (Fig. 2). Contrast and brightness were adjusted with Adobe Photoshop CS5.

To test salt tolerance, strains were grown in 20 ml medium PYEM (2 g peptone, 2 g yeast extract, 0.5 g NH_4Cl, 1 l Milli Q water). After autoclaving and cooling, 5 ml riboflavin (0.2 mg ml^{-1}) sterile filtered, 2 ml 50 % glucose (sterile), 1 ml 20 % $MgSO_4$ (sterile) and 1 ml 10 % $CaCl_2$ (sterile) were added and amended with 0, 5, 10, 20, 30, 40, 60, 80 or 100 g l^{-1} NaCl. The OD_{600} of the cell suspension was determined at the beginning of the experiment and after 2 days. The differences between these two measurements were used to determine salt tolerances. The growth was tested at different temperatures or different pH (pH 5.0, 6.0, 7.0, 8.0, 9.0 or 10.0, adjusted with HCl or KOH; pH readjusted after sterilization) in SPYEM medium with the same OD protocol and the results are given in the species descriptions.

Enzyme activity tests with the use of API ZYM test strips (bioMérieux) and substrate specificity tests with the use of API 20NE and API100 test strips (bioMérieux) were conducted according to the protocol supplied by the manufacturer. The test strips were incubated at 30 °C for 7 days and monitored after 1, 2 and 7 days. A test was considered positive when the interface between sample well and air was visibly turbid due to bacterial growth after a 7-day incubation period. None of the substances in these tests were oxidized by strain MCS23^T or strain MCS27^T; the results are given in the species descriptions. Contrary to all other species of the genus *Henriciella*, strain MCS23^T was positivefor cystine arylamidase. It should be noted here that together with strains MCS23^T and MCS27^T, all species of the genus *Henriciella* are positive for α-glucosidase, although the activity in *H. litoralis* SD10^T is only weak. This discerns *Henriciella* from all other genera of the family *Hyphomonadaceae*.

Of all species of the genus *Henriciella*, cystine arylamidase activity was only observed for MCS23^T. Strain MCS23^T differs from strain MCS27^T by cystine arylamidase activity, the presence of $C_{17:1}\omega5$ and 3-OH $C_{11:0}$, much lower amounts of 3-OH $C_{12:0}$ and absence of $C_{17:1}\omega8$. Strain MCS23^T differs from both *H. marina* and *H. aquimarina* by the presence of a prosthecum, lower amounts of 11-Me-$C_{18:1}\omega5t$ and presence of 3-OH $C_{11:0}$. The differences of MCS27^T to both *H. aquimarina* and *H. marina* are a higher amount of $C_{16:0}$ and 3-OH $C_{12:0}$ and lack of $C_{17:1}\omega5$. Neither MCS23^T nor MCS27^T consume sucrose contrary to both *H. aquimarina* and *H. marina* (Table 2). These characteristics of strains MCS23^T and MCS27^T, together with DNA–DNA-hybridization values below 55 %, are sufficiently different from each other and from the recognized species of the genus *Henriciella* (Table 2) that they are proposed here as *Henriciella barbarensis* sp. nov. (type strain MCS23^T) and *Henriciella algicola* sp. nov. (type strain MCS27^T). Furthermore, the description of the genus *Henriciella* is emended.

EMENDED DESCRIPTION OF THE GENUS *HENRICIELLA* QUAN *ET AL.* 2009

The description of the genus *Henriciella* is as given by Quan *et al.* [29] and emended by Lee *et al.* [12], with the following amendments. Cells of some species possess a stalk, ca. 0.1–0.2 µm in diameter, 0.5–0.7 µm long, varying in length depending on the species and environmental conditions, and extending from one pole as a continuation of the long axis of the cell. Adhesive material is present at the distal end of the stalk. Occur singly. Multiplication by binary fission. For some species at the time of separation, one cell possesses a prosthecum and the other a single polar flagellum. Each new prosthecum occurs at the cell pole opposite to the one formed during fission. The flagellated cell secretes adhesive material at the base of the flagellum, develops a prosthecum at this site and enters the immotile vegetative phase. Colonies are circular, convex and colourless. Chemoorganotrophic, aerobes, can grow anaerobically probably using amino acids as fermentable carbon sources [8]. Cells can store carbon as poly-β-hydroxybutyrate and show activity of α-glucosidase. Species have complex growth requirements and grow on peptone-yeast extract media with 30 g l^{-1} NaCl, with optimal growth between 10–50 g l^{-1} NaCl. No growth occurs with salt concentrations at or below 5 g l^{-1}. The temperature range for growth is 10–40 °C, 20–35 °C optimal. Polar lipids are α-D-glucopyranosyl diacylglycerol, α-D-glucopyranuronosyl diacylglycerol, phosphatidyl diacylglycerol and α-D-glucuronopyranosyl diacylglycerol taurineamide.

DESCRIPTION OF *HENRICIELLA BARBARENSIS* SP. NOV.

Henriciella barbarensis (bar.bar.en′sis. N.L. fem. adj. *barbarensis* derived from a site near to the town Santa Barbara, California).

The description is the same as that given for the genus, with the following additional characteristics. Cells are 1.8 µm

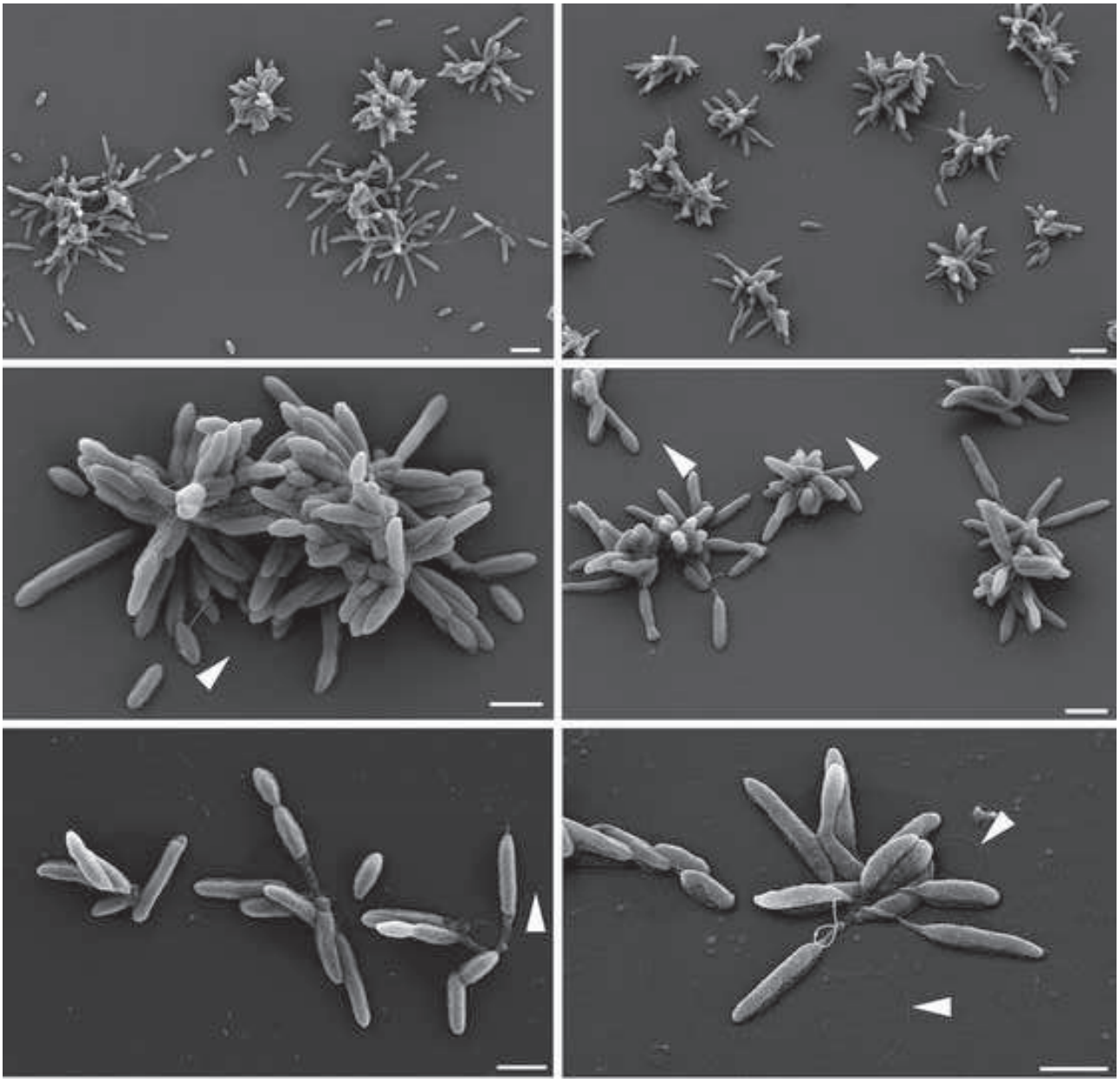

Fig. 2. Scanning electron micrographs of strains MCS23^T (left) and MCS27^T (right) depicting typical growth patterns of prosthecate bacteria in aggregates and connection by stalks (lower images in each row). Arrow heads point to flagella. Bars, 1 µm.

long and 0.3 µm thick, and possess a stalk, 0.7 µm long, depending on growth conditions, with adhesive material at the distal end. Cells reproduce by binary fission and formation of motile swarmer cells with single flagellum. The species is characterized by three major fatty acids, $C_{16:0}$, $C_{17:0}$ and $C_{18:1}\omega7c$, and minor amounts of $C_{11:0}$ 3-OH, $C_{12:0}$ 3-OH, $C_{15:0}$, $C_{18:0}$, $C_{17:1}\omega5$ and $C_{20:1}\omega9$. Can grow on peptone yeast extract media with 5–80 g l^{-1} NaCl with optimal growth between 20 and 80 g l^{-1} NaCl. Growth is observed between 15 and 40 °C and at pH 6–9 with the optimal temperature between 20 and 40 °C. Shows anaerobic growth on SPYEM plates. Does not reduce nitrate, oxidize tryptophan to indole or hydrolyse arginine, urea, aesculin, gelatin or p-nitrophenyl-3-D-galactopyranoside. Cells show activity for alkaline phosphatase, naphthol-AS-BI-phosphohydrolase, leucine arylamidase, acid phosphatase, esterase (C4), esterase lipase (C8), α-glucosidase and cystine arylamidase, and weak activity for trypsin.

The type strain is MCS23^T (=LMG 28705^T=CCUG 66934^T), isolated from filtered seawater from Santa Barbara marine laboratory, University of California. The DNA G+C content of the type strain is 57.0 mol%.

Table 2. Comparison of selected characteristics of species of the genus *Henriciella*

Strains: 1, MCS23^T; 2, MCS27^T; 3, *H. aquimarina* P38^T; 4, *H. litoralis* SD10^T; 5, *H. marina* Iso4^T.

Characteristic	1	2	3	4	5
Colony colour	White	White	Cream white	Yellow	White
Prosthecum	+	+	–	–	–
Growth at 40 °C	+	+	+	+	–
Aesculin hydrolysis	–	–	–	+	–
Enzyme activity					
β-glucosidase	–	–	–	+	–
Cystine arylamidase	+	–	–	–	–
Carbon utilization					
Tween 80	–	–	–	–	+
L-Arabinose	–	–	–	+	+
D-Fructose	–	–	–	+	–
α-D-Glucose	–	–	+	–	–
Raffinose	–	–	–	+	–
Sucrose	–	–	+	–	+
Fatty acids					
$C_{16:0}$	13.4	32.8	21.0	21.2	7.8
$C_{17:1}\omega5$	5.7	–	3.0	7.2	11.7
$C_{17:1}\omega8$	–	3.9	2.7	5.5	–
$C_{18:1}\omega7$	29.3	30.0	38.4	41.2	53.5
3-OH $C_{11:0}$	2.1	–	–	–	–
3-OH $C_{12:0}$	1.1	8.6	4.1	–	1.1
DNA G+C content (mol%)	57.0	55.0	61.0*	55.2*	56.2*

*Data from Lee *et al.* [8].

DESCRIPTION OF *HENRICIELLA ALGICOLA* SP. NOV.

Henriciella algicola [al.gi′co.la. L. fem. *alga* seaweed; L. suff. *-cola* (from L. masc. or fem. n. *incola*)inhabitant; N.L. n. *algicola* inhabitant of algae].

The description is the same as that given for the genus, with the following additional characteristics. Cells are 1.8 µm long and 0.4 µm thick, and possess a stalk, 0.5 µm long, depending on growth conditions, with adhesive material at the distal end. Cells reproduce by binary fission and formation of motile swarmer cells with single flagellum. The species is characterized by three major fatty acids, $C_{16:0}$, $C_{17:0}$ and $C_{18:1}\omega7c$, and minor amounts of $C_{12:0}$ 3-OH, $C_{15:0}$, $C_{17:1}\omega8$, $C_{18:0}$, 11-Me-$C_{18:1}\omega5t$. Can grow on peptone yeast extract media with 5–100 g l^{-1} NaCl with optimal growth between 20 and 100 g l^{-1} NaCl. Growth is observed between 10 and 40 °C and at pH 6–9 with the optimal temperature between 20 and 40 °C. Does not reduce nitrate, oxidize tryptophan to indole or hydrolyse arginine, urea, aesculin, gelatin or p-nitrophenyl-3-D-galactopyranoside. Cells show activities for alkaline phosphatase, naphthol-AS-BI-phosphohydrolase, leucine arylamidase, acid phosphatase, esterase (C4), esterase lipase (C8) and α-glucosidase and weak activity for trypsin but are negative for α- and β-galactosidase, α-glucuronidase, β-glucosidase, α-mannosidase, cystine arylamidase, catalase and α-fucosidase.

The type strain is MCS27^T (=LMG 29152^T=CCUG 67844^T), found growing on *Nannochloris* sp. (*Chlorophyceae*), from Hull Bay, US Virgin Islands. The DNA G+C content of the type strain is 55.0 mol %.

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

Ethical statement
No experiments with humans or animals were performed.

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