Oceanicaulis alexandrii gen. nov., sp. nov., a novel stalked bacterium isolated from a culture of the dinoflagellate *Alexandrium tamarense* (Lebour) Balech

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Five bacterial strains were isolated from a non-toxicogenic strain of the marine dinoflagellate *Alexandrium tamarense* (Lebour) Balech CCMP 116 (NEPCC C116), during a survey of the diversity of bacteria associated with paralytic shellfish toxin-producing cultures of *Alexandrium* spp. (Dinophyta). All strains were strictly aerobic, Gram-negative, straight or curved rods. Cells were dimorphic, with stalks (or prosthecae) and non-motile or non-stalked and motile, by means of a single polar flagellum. The bacteria grew best at salt concentrations ranging from 2 to 10% and growth occurred at 10 °C, but not at 50 °C. The G + C content of the chromosomal DNA of the strains was determined to be 61–62 mol%. Major cellular fatty acids of the bacteria presented a unique profile. 16S rRNA gene sequence analysis showed the five strains to be related to genera of budding bacteria of marine origin in the 'Alphaproteobacteria', namely, *Hirschia*, *Maricaulis* and *Hyphomonas*, although they exhibited substantial differences in morphology, substrate utilization and fatty acid profile to members of these genera. The five strains are proposed to comprise a new species of a new genus, *Oceanicaulis alexandrii* gen. nov., sp. nov., the type strain of which is C116-18^T (=DSM 11625^T =NCIMB 13905^T).

Since the original description of the genus *Caulobacter* by Henrici & Johnson (1935), stalked bacteria from a wide range of sources, including soils, freshwater and marine environments, have been assigned to the genus, mainly on the basis of their characteristic prosthecae (Anast & Smit, 1988; Stahl *et al.*, 1992). Originally thought to be restricted exclusively to oligotrophic habitats, in the last decade an

extensive number of isolates exhibiting heterotrophic traits and relatively versatile metabolisms have been isolated from eutrophic environments, such as secondary waste water treatment facilities (Fenton, 1994; MacRae & Smit, 1991), and assigned to the genus *Caulobacter*. It subsequently became clear, from 16S rRNA gene sequence analyses, that these isolates and the previously described species of *Caulobacter* were genotypically heterogeneous (Stahl *et al.*, 1992), and other studies (Ariskina, 1995; Moore, 1978; Nikitin *et al.*, 1990) supported these findings. A polyphasic analysis (Abraham *et al.*, 1999) of the validly published *Caulobacter* species, members of the phylogenetically related genus *Brevundimonas* and other unpublished caulobacterial isolates yielded a reclassification of many of the described species of *Caulobacter* and the proposal of a new genus, *Maricaulis*, to accommodate the strictly halophilic species. *Caulobacter sensu stricto* is currently composed of only freshwater species and isolates with a specific range of 16S rRNA gene sequence similarity, a characteristic membrane protein antigen and typical polar lipid fingerprints. The amended genus *Brevundimonas* (Abraham *et al.*, 1999), as defined by the same methods,

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The EMBL accession numbers for the 16S rRNA gene sequences of *Oceanicaulis alexandrii* C116-3 and C116-18^T are AJ309863 and AJ309862, respectively.

A selection of 16S rDNA sequence stretches and helices where the five strains of *O. alexandrii* exhibit diagnostic differences from related genera (Table I) and characteristics useful for differentiating the genus *Oceanicaulis* from related genera (Table II) are available in IJSEM Online.

comprises a range of stalked and non-stalked strains from clinical sources, soils, freshwater and marine habitats. Accordingly, species within this genus collectively possess a wide range of phenotypic traits. The genus *Maricaulis* consists of halophilic caulobacteria exclusively of marine origin and is more closely related to the genera of budding marine bacteria, *Hyphomonas* and *Hirschia*, than to *Caulobacter*. In this study, five strains of stalked bacteria isolated from a non-toxicogenic culture of the dinoflagellate *Alexandrium tamarense* are described and a new genus, *Oceanicaulis*, and species, *Oceanicaulis alexandrii*, are proposed which are distinct from previously described stalked bacteria, as their phylogenetic assignment implies.

Bacterial strains were isolated from a culture of *Alexandrium tamarense* (Lebour) Balech CCMP 116 (NEPCC C116), which was collected at Ria de Vigo, Spain, under conditions described previously (Hold *et al.*, 2001). Five isolates were chosen for detailed characterization.

The cultures were routinely maintained on marine agar medium (Difco). Standard methods were used for metabolic testing (Smibert & Krieg, 1981). For fatty acid analysis, the bacteria were grown in marine *Caulobacter* medium (SPYEM), containing 30 g sea salts (Sigma), 0.5 g NH_4Cl and 1 l deionized water, and 20 ml of 50×PYE (100 g peptone, 50 g yeast extract in 1 l deionized water); 2 ml of 50% (w/v) glucose (heat-sterilized) and 5 ml riboflavin (0.2 mg ml^{-1} , filter-sterilized) were added after autoclaving. Testing for oxidation of carbon sources with Biolog GN plates gave poor results. Four strains reacted with Tween 40, two strains were positive for α -hydroxybutyric acid and glycyl-L-glutamic acid, and single strains tested positive for D-raffinose, methylpyruvate, α -ketoglutaric acid, β -hydroxybutyric acid, propionic acid, inosine and uridine. Sample preparation and tests for paralytic shellfish toxins (mouse neuroblastoma assay, HPLC, capillary electrophoresis-mass spectroscopy, ELISA) were carried out as described previously (Gallacher *et al.*, 1997). All five

strains of the novel bacterium tested negative for paralytic shellfish toxins (results not shown). The strains have been deposited at the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany): C116-3, DSM 11627; C116-14, DSM 11669; C116-17, DSM 11626; C116-18^T, DSM 11625^T; C116-20, DSM 11670. Strain C116-18^T has been deposited with the NCIMB (National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland, UK) as NCIMB 13905^T.

PCR amplification of nearly complete 16S rRNA genes (Weisburg *et al.*, 1991) and *Taq*-polymerase-initiated cycle sequencing, using fluorescent-dye-labelled dideoxynucleotides, were carried out as described previously (Abraham *et al.*, 1999). The methodology for alignment and analysis of the resulting DNA sequences has also been reported previously (Abraham *et al.*, 1999).

Comparative sequence analysis revealed two 16S rRNA gene sequence types, which differed in only three nucleotide positions over the nearly complete gene (*Escherichia coli* 16S rRNA gene sequence positions 284, 295, 379), and two (C116-3 and C116-14) and three (C116-17, C116-18^T and C116-20) strains, respectively, were observed to have identical 16S rRNA gene sequences. Comparison of these sequences with those of reference organisms showed the isolates to be phylogenetically related to species of the proposed family 'Rhodobacteraceae' (Garrity *et al.*, 2001) within the 'Alphaproteobacteria' and similar to species of the genera *Maricaulis*, *Hyphomonas* and *Hirschia* (Fig. 1). The five strains were phylogenetically most closely related to the unclassified caulobacterial isolate MCS33 (93.8% sequence similarity), which was isolated from a hydrothermal vent near Vancouver Island, Canada (Abraham *et al.*, 1999). Sequence similarities of strain C116-18^T to the type strains of related genera and species were as follows: *Hyphomonas polymorpha*, 88.1%; *Hyphomonas jannaschiana*, 89.0%; *Hirschia baltica*, 87.3%; *Maricaulis maris* 92.5%; *Maricaulis washingtonensis*, 91.7%; *Maricaulis*

Fig. 1. Unrooted dendrogram based on a comparison of 16S rDNA sequences of strains C116-3 and C116-18^T, and the type species of the genera *Caulobacter*, *Brevundimonas* and *Maricaulis*, within the context of the related 'Alphaproteobacteria'. Sequence data of reference strains were obtained from the GenBank/EMBL and/or RDP databases. In addition to the strains depicted, the 16S rDNA sequences of the following strains have been used to create the dendrogram: *Roseobacter litoralis* ATCC 49566^T (X78312), *Sphingomonas capsulata* ATCC 14666^T (RDP), *Rhizobium radiobacter* ATCC 19358^T (AJ389904), *Rhodospirillum rubrum* ATH 1.1.1 (RDP). Bar, average of 1 nucleotide substitution per 100 nucleotides.

parjimensis, 92.1%; *Caulobacter vibrioides*, 86.0%. Strain and sequence accession numbers used for similarity calculations are given in Fig. 1.

The respective 16S rDNA sequence similarity values of the novel isolates with the species of *Maricaulis*, *Hyphomonas* and *Hirschia*, as well as the type species of *Caulobacter*, *Asticcacaulis* and *Brevundimonas*, indicated that *O. alexandrii* is phylogenetically distinct from the most closely related genera, although many typical sequence patterns are shared with *Maricaulis*. Supplementary Table I (<http://ijs.sgmjournals.org>) provides a selection of 16S rDNA sequence stretches and helices (Neefs *et al.*, 1991) where the five strains of *O. alexandrii* exhibit diagnostic differences from the individual related genera. The sequence patterns listed demonstrate that strains C116-3, C116-4, C-116-17, C116-18^T and C-116-20 are related to the species of the genus *Maricaulis*, although sequence similarity values are lower than what is generally observed between species of a genus. Besides, as the sequence features of *Oceanicaulis* are not restricted to the highly variable regions within the 16S rRNA gene, this is concluded to be sufficient to place *Oceanicaulis* in a distinct genus.

Genomic DNA was isolated from 2 ml of cultures grown in marine broth medium according to the protocol of Wilson (1987), followed by treatment with Ribonuclease A (Sigma) at 50 °C for 2 h, and a phenol/chloroform/isoamyl alcohol (50:40:10) extraction. Appropriate amounts of DNA were enzymically cleaved to yield the nucleosides and the mean G+C contents were determined by HPLC (Tamaoka & Komagata, 1984). Similarity calculations were carried out according to Mesbah *et al.* (1989), with non-methylated λ -phage DNA (Sigma) as a standard.

Cells harvested from 1 ml of liquid culture of each of the five strains studied were lysed by alkaline treatment, according to Birnboim & Doly (1979); screening for plasmids was carried out by electrophoresis on 0.8% agarose/TAE gels. Four of the strains, but not C116-17, showed large plasmids which have not been further analysed. Anast & Smit (1988) reported the occurrence of

large native plasmids (100–150 kb) in two out of 25 marine caulobacteria which they had isolated and studied for their suitability for genetic experiments.

Lipids were extracted, using a modified Bligh–Dyer procedure (Bligh & Dyer, 1959), and fatty acid methyl esters were generated and analysed by GC, as described previously (Vancanneyt *et al.*, 1996).

Fast atom bombardment MS in the negative mode was performed on the first of two mass spectrometers of a tandem high-resolution instrument of E₁B₁E₂B₂ configuration. 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 (Abraham *et al.*, 1997).

Two different types of polar lipids could be detected, i.e. phosphatidyl glycerol and sulfo-quinovosyl diacylglycerol (Fig. 2). Molecular ions were observed at *m/z* 719, 733, 761 and 773, which could be attributed to known phosphatidyl glycerols (Abraham *et al.*, 1997). CID spectra of these ions could not be measured because of their low concentrations in the polar lipid fraction of strain C116-18^T. Strain C116-18^T possessed mainly sulfolipids, while phosphatidyl glycerols were found only in low amounts. With the aid of CID MS, the structures of most of the sulfolipids were elucidated. Diagnostically important fragments were generated by cleavage of diacylglycerol, leading to the dehydrated sulfo-quinovose, which is observed as the (*M*–1)[–] ion at *m/z* 226 (Abraham *et al.*, 1997). CID of the (*M*–H)[–] ion from the sulfolipids yielded neutral losses of the *sn*-2 and *sn*-1 substituent as free carboxylic acid, thus, allowing the identification of the fatty acids attached to the different sulfo-quinovoses. Furthermore, the positions of the fatty acids at the glycerol backbone can be determined because, for the fatty acid positioned at *sn*-2, the neutral loss as free fatty acid is more frequent than for that at *sn*-1 (Heller *et al.*, 1988; Murphy & Harrison, 1994). With this method, the structure of the sulfolipids was identified (Abraham *et al.*, 1997). Table 1 summarizes the results.

Fig. 2. (–)Fast atom bombardment mass spectrum of the total lipid fraction of strain C116-18^T. Between *m/z* 719 and 773, phosphatidyl glycerols are seen; between *m/z* 833 and 877, sulfo-quinovosyl diacylglycerols are seen.

Table 1. Sulfo-quinovosyl diacylglycerols identified in the cell extract of strain C116-18^T

No.	Mass (Da)	R1-COOH (<i>sn</i> -1)	R2-COOH (<i>sn</i> -2)
1	834	17:0	18:1
2	846	18:1	18:1
3	848	18:0	18:1
4	860	19:0	18:2
5	860	19:1	18:1
6	862	18:0	19:1
7	862	18:1	19:0
8	874	19:1	19:1
9	876	19:0	19:1

Polar lipids can be used as a diagnostic feature to discriminate strain C116-18^T from *Maricaulis* and *Hyphomonas* species: C_{18:1}-C_{16:0}-sulfo-quinovosyl glycerol found in all *Maricaulis* strains (Abraham *et al.*, 1997) was not detected in C116-18^T. Furthermore, 1,2-diacyl-3- α -D-glucuronopyranosyl-*sn*-glycerol taurineamide, reported for *Hyphomonas* species (Batrakov *et al.*, 1996) and found in about half of *Maricaulis* strains, was absent.

Strain C116-18^T differed from *Maricaulis* strains by a very low content of C_{16:0} and higher amounts of 7-Me-C_{18:1} ω 6, C_{19:0} and C_{19:1} ω 8. The branched C_{17:0}i found in 4–14% of *Maricaulis* strains is missing (Table 2).

Cells in the late phase of growth were negatively stained

Table 2. Relative amounts of fatty acids of glycolipids and phospholipids found in strain C116-18^T

TR, Trace (<1.0%).

Fatty acid	Glycolipid	Phospholipid
C _{12:0}	TR	
3-OH-C _{12:0}		TR
C _{14:0}	1.0	TR
2-OH-C _{14:0}		TR
C _{16:1} ω 7		TR
C _{16:0}	1.3	1.8
C _{17:0a}	TR	TR
C _{17:1} ω 6	2.9	1.1
C _{17:0}	7.4	9.9
C _{18:2} ω 6,9	TR	TR
C _{18:1} ω 6	0.9	1.8
C _{18:1} ω 7	36.7	27.9
C _{18:0}	16.6	22.1
7-Me-C _{18:1} ω 6	10.9	10.6
C _{19:1} ω 8	7.2	6.9
C _{19:0} d8,9	2.1	2.3
C _{19:0}	10.2	10.4
C _{20:1} ω 9	TR	TR
C _{20:0}		TR

with uranyl acetate (2%, pH 4.5) for analysis by transmission electron microscopy (Fig. 3a, b), as described in detail by Yakimov *et al.* (1998). Negatively stained cells were embedded in epoxy resin (Spurr, 1969) and ultra-thin sections (Fig. 3c) were analysed using an energy-filtered transmission electron microscope (CEM 902; Zeiss), as described in detail by Lünsdorf *et al.* (2001). Upon light and electron microscopy examination, strain C116-18^T was seen as straight or curved motile rods. Further details are given in Fig. 3.

The different caulobacterial genera share a unique cell morphology and mode of cell division. It has been observed previously that caulobacterial strains possess cross walls in the stalks, although, with the exception of strain MCS24, this characteristic had not been observed in marine caulobacteria. Anast & Smit (1988) have already noted that MCS24 is tolerant of freshwater conditions, and a phylogenetic analysis (Abraham *et al.*, 1999) revealed it to be a member of the genus *Brevundimonas* and not of *Maricaulis*, a genus of exclusively marine origin. For the first time, stalk cross walls have been observed in a caulobacterium which is phylogenetically affiliated with the caulobacteria of marine origin in the genus *Maricaulis*. This is a morphological feature which separates the C116- strains from all species of *Maricaulis* examined so far.

Most caulobacteria require complex organic additives, such as peptone, in their growth media (Poindexter, 1964), which complicates their nutritional characterization. Therefore, the description of the five new strains as a new genus and species given below is mainly based on their characteristic lipid composition, their distinct 16S rDNA sequences and morphological differences to related genera. Characteristics useful for differentiating the genus *Oceanicaulis* from related genera can be found in Supplementary Table II (<http://ijs.sgmjournals.org>).

Description of *Oceanicaulis* gen. nov.

Oceanicaulis [O.ce.an.i.cau'lis. L. masc. n. *oceanus* the ocean; L. masc. n. *caulis* stalk, referring to a prostheca; N.L. masc. n. *Oceanicaulis* stalk(ed organism) from the ocean].

Gram-negative, non-spore-forming. Cells are rod-shaped or vibrioid. Cultivated cells are 1 μ m by 3 μ m. Cells possess one prostheca, which extends centrally along the long cell axis from one pole. Adhesive material is present at the distal end of the prostheca. During binary fission, at the point of separation, one cell possesses a prostheca and the other a single polar flagellum. Colonies are round, convex and non-pigmented. Aerobic and chemo-organotrophic. Nitrate is reduced to nitrite by most strains. All strains grow with 20–100 g NaCl l⁻¹. Mesophilic. Polar lipids are phosphatidyl glycerol and sulfo-quinovosyl diacylglycerol. Major fatty acids are C_{18:1} ω 7, C_{18:0}, 7-Me-C_{18:1} ω 6 and C_{19:0}. The G + C content of the DNA is 61–62 mol%. Phylogenetically, the genus is related to the prosthecate bacteria of

Fig. 3. Electron micrographs of strain C116-18^T. (a) The two morphologies observed for strain C116-18^T. First, a motile swarmer cell with monotrichous and monopolar flagellation (white arrowheads) and, second, a non-motile, stalked cell in a state of binary fission (arrow). Note the holdfast at the end of the stalk (black arrowhead) (Anast & Smit, 1988; Merker & Smit, 1988; Poindexter & Cohen-Bazire, 1964; Umbreit & Pate, 1978). (b) A more detailed view of a stalk. The interior of the stalk contains granularly shaped microbodies (white arrowheads), which are distributed equally throughout the stalk lumen. Since these microbodies (twin-arrowheads) are able to traverse the constriction site (arrow), this feature does not seem to represent a septum-like arrangement, but rather an open ring. In consequence, the stalk is an uncompartimentalized, continuous tube. This type of constriction is found widely among freshwater caulobacteria (Anast & Smit, 1988). Additionally, the stalk surface is covered by a regularly arranged protein S-layer (see inset, arrowheads indicate the protein interspacings). (c) Ultra-thin sections of isolate C116-18^T exhibited a Gram-negative cell wall architecture, with a darkish outermost line, representing the outer membrane (black arrowheads). Between the outer membrane and the cytoplasm, the periplasmic space appears relatively electron-translucent and seems to be in direct contact with the lumen of the stalk (arrow).

marine origin, namely, *Maricaulis*, *Hyphomonas* and *Hirschia*, within the order 'Rhodobacterales'.

The type species is *Oceanicaulis alexandrii*.

Description of *Oceanicaulis alexandrii* sp. nov.

Oceanicaulis alexandrii [a.lex.an.dri'i. L. n. *Alexandrium* the (dinoflagellate) from Alexandria; L. masc. gen. *alexandrii* of *Alexandrium*, the source of isolation and postulated natural habitat].

The stalk of prosthecate cells possesses a characteristic constriction. In addition to the major fatty acids typical for the genus, the following minor fatty acids are present: C_{14:0}, C_{16:0}, C_{17:1ω6}, C_{17:0}, C_{18:1ω6}, C_{19:1ω8}, C_{19:0d8,9}. Optimal growth occurs at 30 °C, with a growth range from 4 to 37 °C. Cells grow in marine broth at full strength and at 1/10 strength. Strains are catalase- and oxidase-positive. Strains tested negative for lecithinase, starch, xanthine, indole, *o*-nitrophenylgalactoside and Voges–Proskauer, and

most tested negative for aesculin, elastinase, gelatinase and lipase. Most strains contain large plasmids.

The type strain is C116-18^T (=DSM 11625^T=NCIMB 13905^T). The G+C content of its genomic DNA is 61.8 mol%.

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