Phylogeny of *Maricaulis* Abraham *et al.* 1999 and proposal of *Maricaulis virginensis* sp. nov., *M. parjimensis* sp. nov., *M. washingtonensis* sp. nov. and *M. salignorans* sp. nov.

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Thirteen marine bacteria isolated from different locations, belonging to the genus *Maricaulis*, were characterized by 16S rRNA gene sequencing, DNA–DNA hybridizations and analysis of the internally transcribed 16S–23S rDNA spacer (ITS1) region, analysis of fatty acids from total lipids, mass spectrometry of polar lipids and determination of temperature and NaCl tolerances. The data obtained led to the identification of five new sulfoquinovosyl diacylglycerols, using tandem mass spectrometry, and the fragmentation patterns of the individual compounds. Four novel species were identified and described as *Maricaulis virginensis* sp. nov. (type strain VKM B-1513^T), *Maricaulis parjimensis* sp. nov. (type strain MCS 25^T), *Maricaulis washingtonensis* sp. nov. (type strain MCS 6^T) and *Maricaulis salignorans* sp. nov. (type strain MCS 18^T). They differ in their temperature and salt tolerances and can be differentiated by their polar lipids and fatty acid patterns, as well as their ITS1 and 16S rRNA gene sequences.

Keywords: *Maricaulis virginensis*, *Maricaulis parjimensis*, *Maricaulis washingtonensis*, *Maricaulis salignorans*, sulfolipid

INTRODUCTION

Caulobacteria are dimorphic, prosthecate bacteria in which reproduction results in the separation of two cells that are morphologically and behaviourally distinct from each other. One of these cells is sessile, being non-motile, and prosthecate, possessing at least one elongated, cylindrical appendage, a prostheca (Staley, 1968). The other cell is flagellated, bearing one polar flagellum, by which it is motile. The mode of reproduction of the dimorphic, prosthecate bacteria is unique as a regular feature of a prokaryotic repro-

ductive cycle. It helps to disperse the population at each generation, thereby minimizing competition among siblings for resources. These developmental and reproductive characteristics allow these bacteria to exist in oligotrophic habitats (Poindexter, 1981a), importantly, tolerating prolonged nutrient scarcity.

Caulobacteria are ecologically important because they are ubiquitous in water and are presumed to be responsible for considerable mineralization of dissolved organic material in aquatic environments, with the oligotrophic caulobacters being especially important when nutrient concentrations and ambient temperatures are low (Staley *et al.*, 1987). Studies have revealed that practically any type of sea water contains caulobacteria (Jannasch & Jones, 1960; Anast & Smit, 1988).

The first isolation of a *Caulobacter* sp. was reported by Löffler (1890), from fresh water. He noticed the unusual nutrient requirements of the strain and he could not assign the strain to a known taxon. In 1935, Henrici and Johnson described the genus *Caulobacter*,

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Abbreviations: ARDRA, amplified rDNA restriction analysis; CID, collision-induced dissociation; FAB, fast atom bombardment; FAME, fatty acid methyl ester; ITS, internally transcribed spacer; PYE, peptone/yeast extract; SQDG, sulfoquinovosyl diacyl glycerol; SSCP, single-stranded conformational polymorphism.

The GenBank accession numbers for the 16S rDNA sequences of strains MCS 10, MCS 30, MCS 31 and VKM B-1513^T are respectively AJ301664–AJ301667.

with *Caulobacter vibrioides* as the type species (Henrici & Johnson, 1935). Three decades later, Poindexter (1964) described *Caulobacter maris* and *Caulobacter halobacteroides*, the first two caulobacteria from sea water. At that time, the taxonomy of the members of the genus was based almost exclusively on the characteristic morphology and mode of reproduction (Poindexter, 1981b).

Anast & Smit (1988) were the first to determine differences between freshwater and marine caulobacteria on a large set of strains. Stahl *et al.* (1992) sequenced the 16S rRNA of several isolates belonging to *Caulobacter*, revealing, for the first time, that members of *Caulobacter* actually formed two distinct lineages, one comprising the freshwater and brackish water *Caulobacter* strains and the other comprising most of the marine *Caulobacter* strains.

As caulobacteria are ubiquitous in water samples and are believed to play an important role in carbon cycling in their habitats, we became interested in this group of bacteria and evaluated the diversity of freshwater and marine *Caulobacter*, using lipid analysis (Abraham *et al.*, 1997), immunological profiling and 16S rRNA gene sequences and compared these results with physiological data (Abraham *et al.*, 1999). We observed all freshwater caulobacteria to belong to the genera *Caulobacter* and *Brevundimonas*. However, the truly marine, halophilic caulobacteria belong to a separate genus, which we named *Maricaulis*, consisting of a single species, *Maricaulis maris* (Abraham *et al.*, 1999). We report here the characterization of a number of marine isolates, obtained from different habitats from a wide range of locations, that belong to this genus and propose four novel species.

METHODS

Strains. The strains used in this study were obtained from the ATCC, the All-Russian Collection of Microorganisms (VKM), the DSMZ and the Dept of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada (VC and MCS strains). For the origin of the isolates, see Table 1.

All strains were grown in marine-*Caulobacter* medium SPYEM containing 30 g sea salts (Sigma) and 0.5 g NH₄Cl in 1 l deionized water. After autoclaving and cooling, 20 ml 50 × PYE, 2 ml 50% glucose (sterile) and 5 ml riboflavin (0.2 mg ml⁻¹), filter-sterilized, were added (50 × PYE is 100 g peptone and 50 g yeast extract in 1 l deionized water, autoclaved). The strains were grown in 2 l flasks, at 30 °C and 100 r.p.m. and biomass was harvested in the late exponential phase after 72 h by centrifugation at 5860 *g* at room temperature.

Genomic DNA base composition analysis. Genomic DNA was isolated from cell pellets according to the protocol of Wilson (1987), followed by treatment with RNase A (Sigma) at 50 °C for 2 h and additional phenol/chloroform/isoamyl alcohol extraction. DNA was enzymically digested and mean G + C contents were determined by HPLC (Tamaoka & Komagata, 1984). Calculations were carried out according to Mesbah *et al.* (1989), using non-methylated lambda phage DNA (Sigma) as a standard.

16S rDNA sequencing. Genomic DNA for PCR targeting the 16S rRNA genes (Mullis & Faloona, 1987) was prepared from individual colonies picked from agar media, as described previously (Moore *et al.*, 1997). *Taq* polymerase-initiated cycle sequencing with standard primers was carried out as described previously (Abraham *et al.*, 1999, 2001). The sequence data were aligned with reference 16S rRNA and 16S rRNA gene sequences (Maidak *et al.*, 2001; Stoesser *et al.*, 2001), using the evolutionarily conserved primary sequence and secondary structure (Gutell *et al.*, 1985; Neefs *et al.*, 1991) as references, and analysed using the programs of the PHYLIP package (Felsenstein, 1989), as described previously (Abraham *et al.*, 1999, 2001).

DNA–DNA hybridization. DNA was isolated according to the protocol of Marmur (1961). DNA–DNA hybridizations were performed using a modification of the microplate method described by Ezaki *et al.* (1989), using an HTS7000 Bio Assay Reader (Perkin Elmer) for the fluorescence measurements. Biotinylated DNA was hybridized with single-stranded unlabelled DNA, non-covalently bound to microplate wells. Hybridizations were performed at 48 °C in hybridization mixture (2 × SSC, 5 × Denhardt's solution, 2.5% dextran sulfate, 50% formamide, 100 µg denatured salmon sperm DNA ml⁻¹, 1250 ng biotinylated probe DNA ml⁻¹).

Rapid analysis based on internally transcribed 16S–23S rDNA spacer (ITS1) regions. A rapid DNA isolation method from bacterial colonies was used (Guasp *et al.*, 2000). The ITS1 regions from strains MCS 25^T, MCS 26, VKM B-1513^T and VKM B-1514 were amplified by PCR as described above and using primers designed to anneal at conserved positions in the 3' and 5' regions, respectively, of the bacterial 16S rRNA and 23S rRNA genes (Abraham *et al.*, 1999; Guasp *et al.*, 2000). Amplified rDNA restriction analysis (ARDRA) of the ITS1 PCR products was performed by digestion with the tetrameric endonuclease *TaqI* (MBI Fermentas) according to the conditions described elsewhere (Abraham *et al.*, 1999). The existence of possible heterogeneities for each resulting band of the ARDRA fingerprints was examined by single-stranded conformational polymorphism (SSCP), exactly as described previously (Abraham *et al.*, 1999).

Whole-cell fatty acid analysis. Cells were saponified, methylated to fatty acid methyl esters (FAMES) and extracted as described in detail by Osterhout *et al.* (1991). FAMES were analysed on a Hewlett Packard 5890A gas chromatograph. Separation of FAMES 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 Ultra2). The computer-controlled parameters were the same as those described by Osterhout *et al.* (1991). The instrument was equipped with a flame-ionization detector and an auto-sampler (HP 7673). H₂ served as carrier gas.

Polar lipid fatty acid analysis

Lipids were extracted using a modified Bligh–Dyer procedure (Bligh & Dyer, 1959) as described previously (Vancanneyt *et al.*, 1996). FAMES were generated and analysed by GC and GC-MS as described previously (Abraham *et al.*, 1999).

¹H NMR spectra were recorded in 7:3 d-chloroform/d₄-methanol at 300 K on a Bruker ARX-400 NMR spectrometer relative to internal tetramethylsilane using the standard Bruker software.

Fast atom bombardment (FAB)-MS. FAB-MS in the positive and negative modes was performed on the first of two mass spectrometers of a tandem high-resolution instrument in an

Table 1. Strains used in this study and their origins

Strain	Described here as:	Origin
<i>M. maris</i> ATCC 15268 ^T		Filtered sea water
<i>M. maris</i> ATCC 15269*		Filtered sea water
MCS 25 ^T	<i>Maricaulis parjimensis</i> sp. nov. MCS 25 ^T	Filtered sea water, Indian Ocean off Goa, India
MCS 18 ^T	<i>Maricaulis salignorans</i> sp. nov. MCS 18 ^T	Sea water, Salsbury Point County Park, adjacent to Hood Canal Bridge, WA, USA
MCS 10	<i>Maricaulis</i> sp. MCS 10	Filtered sea water, Anacortes, WA, USA
MCS 11	<i>Maricaulis</i> sp. MCS 11	Filtered sea water, Anacortes, WA, USA
MCS 26	<i>Maricaulis</i> sp. MCS 26	Filtered sea water, Indian Ocean off Goa, India
MCS 28	<i>Maricaulis</i> sp. MCS 28	Filtered sea water, Burrard Inlet, Vancouver, British Columbia, Canada
MCS 30	<i>Maricaulis</i> sp. MCS 30	Sechelt Peninsula, near Egmont, British Columbia; scraping from salmon aquaculture net treated with tributyl tin oxide paint
MCS 31	<i>Maricaulis</i> sp. MCS 31	Sechelt Peninsula, Wood Bay, British Columbia; from a sea-water sample taken from a salmon aquaculture facility
VKM B-1513 ^T	<i>Maricaulis virginensis</i> sp. nov. VKM B-1513 ^T	Deep sea, Virgin Islands (J. Poindexter, strain VC-5 ^T)
VKM B-1514	<i>M. virginensis</i> sp. nov. VKM B-1514	Deep sea, Virgin Islands (J. Poindexter, strain VC-13)
MCS 6 ^T	<i>Maricaulis washingtonensis</i> sp. nov. MCS 6 ^T	Sea water, Inner Marina, Edmonds, WA, USA

* Type strain of *C. halobacteroides*, a heterotypic synonym of *M. maris* (Abraham *et al.*, 1999).

E₁B₁E₂B₂ configuration (JMS-HX/HX110A, JEOL Tokyo) 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.

Tandem MS. 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.

Phenotypic characterization. Strains were grown in 20 ml medium PYEM (2 g peptone, 2 g yeast extract, 0.5 g NH₄Cl, 1 l Milli-Q water). After autoclaving and cooling, 5 ml riboflavin (0.2 mg ml⁻¹), filter-sterilized, 2 ml 50 % glucose (sterile), 1 ml 20 % MgSO₄ (sterile) and 1 ml 10 % CaCl₂ (sterile) were added, amended with 0, 5, 10, 20, 30, 40, 60, 80 or 100 g NaCl l⁻¹. The OD₅₄₀ 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. Growth was tested at different temperatures in SPYEM medium with the same protocol.

RESULTS

DNA G + C content

The G + C contents of the DNA of strains MCS 25^T, MCS 18^T and MCS 6^T were between 63.0 and 63.3 mol %. This is close to the value of 62.6 mol %

determined for *M. maris* ATCC 15268^T (Abraham *et al.*, 1999). The G + C content for strain VKM B-1513^T was somewhat higher, 65.2 mol %.

16S rDNA sequencing

Analysis of the nearly complete 16S rDNA sequences of the strains investigated in this study revealed three distinct phylogenetic lineages within the genus *Maricaulis*. The first 16S rDNA lineage consisted of the type species of the genus, *M. maris*, together with five additional sea-water strains (Fig. 1). The second lineage consisted of a cluster of four strains, MCS 25^T, MCS 26, VKM B-1513^T (= VC-5^T) and VKM B-1514 (= VC-13), while the third lineage was formed by the two closely related strains MCS 6^T and MCS 18^T.

The four organisms in the second lineage, like the two strains in the third lineage, possessed highly similar 16S rDNA sequences. Strains VKM B-1513^T and VKM B-1514 shared identical 16S rDNA sequences, which were respectively 99.9 and 99.6 % similar to the 16S rDNA sequences of MCS 26 and MCS 25^T. The sole nucleotide difference between the sequences of strains VKM B-1513^T and VKM B-1514 and that of strain MCS 26 was situated in helix 6, where strain MCS 26 possessed two additional nucleotides in the stem of the helix. The 16S rDNA sequences of strains VKM B-1513^T, VKM B-1514 and MCS 26 possessed 96.3 % similarity to the 16S rDNA sequence of *M. maris* ATCC 15268^T, while strain MCS 25^T showed 96.6 % sequence similarity to the type species of the genus. The type strain of the type species of *Hyphomonas*, *Hyphomonas polymorpha* DSM 2665^T, the genus most closely related to *Maricaulis*, revealed

Fig. 1. Unrooted dendrogram based on a comparison of 16S rDNA sequences of strains belonging to the genus *Maricaulis* within the context of the subclass α -Proteobacteria. Sequence data for strains of other genera were obtained from the GenBank/EMBL (Stoesser *et al.*, 2001) and/or RDP (Maidak *et al.*, 2001) databases. Accession numbers are given in parentheses. *Comamonas testosteroni* (RDP) was used as an outgroup (not shown). Bar, 1 nucleotide substitution per 100 bases.

sequence similarity values ranging from 90.2 to 90.5% to the sequences of the strains studied. Within the third 16S rDNA lineage, strains MCS 6^T and MCS 18^T shared 99.9% 16S rDNA sequence identity in 1422 homologous nucleotide positions analysed, while the sequence similarities to the most similar 16S rDNA of the validly published species *M. maris* were respectively 96.3 and 96.2%. Strains MCS 6^T and MCS 18^T differed in only one nucleotide, at position 156 (*Escherichia coli* numbering) in helix 9 (Neefs *et al.*, 1991), where a cytosine in the sequence of MCS 6^T was replaced by a thymidine in the sequence of MCS 18^T. This substitution resulted in a G–T pairing in the secondary structure of helix 9 of MCS 18^T, instead of a G–C pair in the secondary structure of MCS 6^T. The 16S rDNA

Fig. 2. Fingerprints generated after *TaqI* restriction analysis of PCR-amplified ITS1 of the different strains of *Maricaulis* in 3.0% agarose gels. Lanes: M, 1 kb DNA molecular mass ladder (Gibco); 1, *Maricaulis* strain VKM B-1513^T (=VC-5^T); 2, *Maricaulis* strain VKM B-1514 (=VC-13); 3, *Maricaulis* strain MCS 25^T; 4, *Maricaulis* strain MCS 26.

similarity values of these two strains to strains of the second lineage ranged from 94.3 to 94.7%.

DNA–DNA hybridization

The results of DNA–DNA hybridizations of *Maricaulis* strains are listed in Table 2. Strains VKM B-1513^T and VKM B-1514 were observed to be highly related (83%) and both strains showed close relatedness to strain MCS 26 (70%). Strain MCS 25^T is separated from all other strains. As described previously (Abraham *et al.*, 1999), *M. maris* (*C. maris*) ATCC 15268^T and *C. halobacteroides* ATCC 15269^T are highly related (92%) and their names are

Table 2. DNA–DNA hybridization of *Maricaulis* strains

Values for DNA–DNA hybridization are means with standard deviations given in parentheses. ND, Not determined.

Strain	G + C content (mol %)	DNA–DNA hybridization with DNA from:							
		1	2	3	4	5	6	7	8
1. <i>M. maris</i> ATCC 15268 ^T	63.3	100							
2. <i>M. maris</i> ATCC 15269	63.2	92	100						
3. <i>Maricaulis</i> sp. MCS 25 ^T	63.0	23 (1)	ND	100					
4. <i>Maricaulis</i> sp. MCS 26	64.9	ND	ND	28 (13)	100				
5. <i>Maricaulis</i> sp. VKM B-1513 ^T	65.2	ND	ND	32 (11)	70 (16)	100			
6. <i>Maricaulis</i> sp. VKM B-1514	65.3	33 (13)	27 (17)	28 (4)	ND	83 (11)	100		
7. <i>Maricaulis</i> sp. MCS 6 ^T	63.0	ND	ND	21 (6)	25 (5)	25 (2)	24 (5)	100	
8. <i>Maricaulis</i> sp. MCS 18 ^T	63.1	32 (15)	19 (15)	18 (3)	17 (8)	23 (2)	19 (1)	59 (9)	100

Table 3. Fatty acid content of whole-cell hydrolysates of *Maricaulis* strains

Values are percentages of total fatty acids. Strains: 1, *Maricaulis* sp. MCS 10; 2, *Maricaulis* sp. MCS 11; 3, *Maricaulis* sp. MCS 28; 4, *M. maris* ATCC 15268^T; 5, *M. maris* ATCC 15269; 6, *Maricaulis* sp. MCS 26; 7, *M. virginensis* sp. nov. VKM B-1513^T; 8, *M. virginensis* sp. nov. VKM B-1514; 9, *M. parjimensis* sp. nov. MCS 25^T; 10, *M. salignorans* sp. nov. MCS 18^T; 11, *M. washingtonensis* sp. nov. MCS 6^T. Strains are listed in the order of Fig. 1. Only fatty acids accounting for more than 1.0% (mean amount) of the total fatty acids are indicated. tr, Trace amount (< 1.0%); —, not detected.

Fatty acid	1	2	3	4	5	6	7	8	9	10	11
11:0 iso 3OH	2.1	2.8	2.0	2.6	3.8	1.6	5.4	5.0	tr	tr	tr
12:0 3OH	—	—	—	—	—	tr	—	—	—	—	—
15:0	tr	tr	tr	tr	tr	—	—	—	—	tr	tr
16:0	14.0	14.0	13.0	17.0	16.0	7.6	9.8	12.3	3.6	8.9	11.0
Summed feature 4*	3.0	3.2	3.2	6.6	6.0	1.9	2.4	3.9	2.2	2.6	3.0
16:1 ω 9c	tr	tr	tr	1.0	tr	—	—	—	—	tr	tr
17:0	11.0	11.0	14.0	5.3	6.2	11.6	15.3	13.7	7.0	8.7	9.0
17:1 ω 6c	1.7	1.0	1.6	tr	tr	3.2	1.3	—	1.8	1.1	1.0
17:1 ω 8c	6.6	6.6	8.3	4.0	4.8	8.0	9.6	10.0	4.7	10.0	10.0
17:0 iso	12.0	6.7	11.1	7.7	7.1	4.8	6.9	5.4	1.7	10.8	9.6
17:1 iso ω 9c	18.9	16.7	19.0	17.4	16.9	6.1	13.8	13.5	3.9	28.0	22.4
18:0	1.0	1.4	1.0	1.1	1.1	4.6	4.2	3.8	7.9	tr	tr
Summed feature 7	19.0	25.6	17.2	24.5	24.0	33.1	13.0	18.1	47.9	12.9	16.2
18:1 ω 9c	4.5	5.4	4.1	6.4	6.2	5.1	3.4	4.2	6.0	7.7	10.7
ECL 18:080†	tr	tr	tr	1.6	1.6	1.4	2.9	2.1	tr	1.0	tr
ECL 18:424	1.9	1.9	1.9	1.3	1.3	5.4	5.7	4.2	2.3	1.8	1.3
ECL 18:797	tr	tr	tr	tr	tr	3.9	2.6	2.2	4.9	tr	tr

* Summed features consist of one or more fatty acids that could not be separated by the Microbial Identification System. Summed feature 4: 15:0 iso 2OH, 16:1 ω 7c and/or 16:1 ω 7t. Summed feature 7: 18:1 ω 7c, 18:1 ω 9t and/or 18:1 ω 12t.

† Unidentified fatty acids with equivalent chain length (ECL) given.

synonyms, and they are not related to any of the other strains studied.

ITS1 analysis

ITS1 analysis was applied as a rapid bacterial identification and typing tool in order to confirm the close relatedness among four *Maricaulis* strains. The utility of ITS1 for the clarification of the phylogenetic positions of these *Maricaulis* strains has been demonstrated by the ability of these nucleic acid regions to differentiate closely related organisms at the species level (Guasp *et al.*, 2000). The strains submitted to this high-resolution analysis were strains MCS 26, MCS 25^T, VKM B-1513^T (= VC-5^T) and VKM B-1514 (= VC-13). For all strains, a single ITS1 PCR product was obtained. Agarose gel electrophoresis mobility differences between the strains were not clearly detected after this first step (data not shown). Each of the four strains displayed a single agarose gel band corresponding to a PCR product of between 1.6 and 2.0 kb, of which approximately 0.6 kb comprises the 16S rDNA 3'-region and nearly 0.5 kb comprises the 23S rDNA 5'-region; the difference (~ 0.5 to ~ 0.9 kb) comprises the ITS1 regions. As a second step, the individual ITS1 PCR products of the four strains were subjected to *TaqI* ARDRA presumptive fingerprint differentiation analysis. Strains MCS 26, VKM B-1513^T and VKM B-1514 showed identical ARDRA

patterns, confirming a close phylogenetic relationship among these three strains (Fig. 2). A clearly distinct fingerprint was observed for strain MCS 25^T, sharing only two bands with the fingerprints observed for the remaining strains (Fig. 2). These results were supported by SSCP for evaluation of the existence of sequence heterogeneities for each restriction band observed for strains sharing the same ARDRA pattern, i.e. MCS 26, VKM B-1513^T (= VC-5^T) and VKM B-1514 (= VC-13). Identical SSCP band patterns were obtained for strains VKM B-1513^T and VKM B-1514, while some bands in the SSCP pattern for strain MCS 26 showed subtle differences in mobility, directly related to differences in single-strand conformations in comparison with strains VKM B-1513^T and VKM B-1514 (data not shown).

Analysis of FAMES

The cellular fatty acid compositions of *Maricaulis* type strains and related strains are shown in Table 3. Strains VKM B-1513^T and VKM B-1514 possessed considerable amounts of 11:0 iso 3OH, a fatty acid that was found in only trace amounts in strains MCS 18^T and MCS 6^T. Strains MCS 26 and MCS 25^T differed from all other *Maricaulis* strains analysed so far in possessing higher contents of the unidentified fatty acids ECL 18:424 and ECL 18:797. Strains MCS 18^T and MCS 6^T possessed the largest amounts of all

Table 4. Glycolipids identified in *Maricaulis* strains by (–)-FAB CID-MS

Mass	Type*	Compound	Strain(s)
730	MGDOx	1-C16:0-2-C15:0-glucuronyl-glycerol	MCS 31
742	MGDOx	1-C16:0-2-C16:1-glucuronyl-glycerol	MCS 28
744	MGDOx	1-C15:0-2-C17:0-glucuronyl-glycerol	MCS 31
744	MGD	1-C17:0-2-C16:0-glucosyl-glycerol	MCS 31
756	MGDOx	1-C17:1-2-C16:0-glucuronyl-glycerol	MCS 28
766	MGDOx	1-C18:1-2-C16:2-glucuronyl-glycerol	MCS 28
770	MGDOx	1-C18:1-2-C16:0-glucuronyl-glycerol	MCS 28
770	MGD	1-C19:1-2-C16:0-glucosyl-glycerol	MCS 28
770	MGDOx	1-C17:1-2-C17:0-glucuronyl-glycerol	MCS 28
770	MGD	1-C17:0-2-C18:1-glucosyl-glycerol	MCS 28
772	MGDOx	1-C18:0-2-C16:0-glucuronyl-glycerol	MCS 28
780	MGDOx	1-C17:2-2-C18:1-glucuronyl-glycerol	MCS 28
780	MGDOx	1-C17:1-2-C18:2-glucuronyl-glycerol	MCS 28
780	MGD	1-C18:2-2-C18:1-glucosyl-glycerol	MCS 28
782	MGDOx	1-C17:1-2-C18:1-glucuronyl-glycerol	MCS 6 ^T , MCS 28
784	MGDOx	1-C19:1-2-C16:0-glucuronyl-glycerol	MCS 28
784	MGDOx	1-C18:0-2-C17:1-glucuronyl-glycerol	MCS 28
784	MGDOx	1-C18:1-2-C17:0-glucuronyl-glycerol	MCS 28
796	MGDOx	1-C18:1-2-C18:1-glucuronyl-glycerol	MCS 28
798	MGDOx	1-C18:1-2-C18:0-glucuronyl-glycerol	MCS 10
798	MGD	1-C19:0-2-C18:1-glucosyl-glycerol	MCS 10
812	MGDOx	1-C19:0-2-C18:1-glucuronyl-glycerol	MCS 28

* MGD, 1,2-Diacyl-glucosyl-glycerol; MGDOx, 1,2-diacyl-glucuronyl-glycerol.

Maricaulis strains studied of 17:1 ω 8c and 17:1 iso ω 9c.

MS analysis of polar lipids

A number of unique glycolipids were characterized from the polar lipid fraction of *Maricaulis* strains by MS and ¹H NMR. The NMR spectra revealed that the glycolipids all belong to the α -glucosyl and α -glucuronosyl diacyl glycerol types. The structures of the individual compounds were elucidated by FAB- and CID-MS in a tandem mass spectrometer. The mass-spectrometric behaviour of these compounds in the negative ionization mode strongly resembles that of phospholipids (Murphy & Harrison, 1994; Abraham *et al.*, 1997). They show distinct [M–H][–] ions. CID of [M–H][–] ions yields abundant carboxylate anions from both the *sn*-1 and *sn*-2 position of the glycerol backbone, allowing the identification of the glycolipid species. The relative abundance of the carboxylate anions may provide evidence for the relative positions of the two acyl functions. By analogy to phospholipids, the loss of the *sn*-2-acyl position may be favoured, thus yielding a more abundant carboxylate anion (Murphy & Harrison, 1994). In the *Maricaulis* strains studied, α -glucuronosyl diacyl glycerols are more abundant and, as can be seen by the compounds identified, exist in a broader variety than the α -glucosyl diacyl glycerols (Table 4).

The polar lipids of *Maricaulis* strains VKM 1513^T and VKM 1514 consist mainly of sulfolipids (Table 5). They were identified by MS as sulfoquinovosyl diacyl glycerols (SQDG) (Abraham *et al.*, 1997). Besides the known compounds 1–3 (Table 6), strains VKM 1513^T and VKM 1514 also contained novel SQDGs in the mass range *m/z* 848–862. They were identified as compounds 4–8 (Table 6) by using tandem MS and the fragmentation patterns described previously (Abraham *et al.*, 1997).

The patterns of polar lipids reveal two groups of *Maricaulis* strains, one comprising the type species, *M. maris*, and strains MCS 6^T, MCS 10, MCS 11, MCS 18^T, MCS 28, MCS 30 and MCS 31, characterized by a number of different glycolipids, sometimes together with taurineamides (Batrakov *et al.*, 1996), while the other group, containing *Maricaulis* strains MCS 25^T, MCS 26, VKM B-1513^T and VKM B-1514, has large amounts of sulfolipids of the SQDG type.

Phenotypic characterization

All *Maricaulis* strains grew best with salt concentrations between 20 and 80 g NaCl l^{–1}. *Maricaulis* strain MCS 18^T also grew without salt, but with a slightly reduced growth rate. *Maricaulis* strain MCS 6^T was more sensitive than strain MCS 18^T to high salt concentrations and showed reduced growth at salt

Table 5. Polar lipids identified by MS in *Maricaulis* strains

Strains: 1, *Maricaulis* sp. MCS 10; 2, *Maricaulis* sp. MCS 11; 3, *Maricaulis* sp. MCS 28; 4, *Maricaulis* sp. MCS 30; 5, *M. maris* ATCC 15268^T; 6, *M. maris* ATCC 15269; 7, *Maricaulis* sp. MCS 31; 8, *Maricaulis* sp. MCS 26; 9, *M. virginensis* sp. nov. VKM B-1513^T; 10, *M. virginensis* sp. nov. VKM B-1514; 11, *M. parjimensis* sp. nov. MCS 25^T; 12, *M. salignorans* sp. nov. MCS 18^T; 13, *M. washingtonensis* sp. nov. MCS 6^T. Strains are listed in the order of Fig. 1.

Mass	Type*	Fatty acid		Detected in strain:†												
		R ₁ COOH	R ₂ COOH	1	2	3	4	5	6	7	8	9	10	11	12	13
720	PG	18:1	14:0			F		F								
730	GL									C						
734	PG	18:1	15:0					F								
742	GL				F	C										C
744	GL									C						
746	PG	18:1	16:1						F							
748	PG	18:1	16:0			F		F	F							
750	PG	19:0	15:0					F								
756	GL			F	F	C	F			F					F	F
762	PG	19:1	16:0					F								
768	GL				F											
770	GL			F	F	C	F			F		C			F	F
772	GL					C						F			F	
774	PG	18:1	18:1			C			F							
780	GL					C										
782	GL			F	F	C							F			C
784	GL			F	F	C	F	F	F	F		C	F		F	F
796	GL			F	F	C									F	F
798	GL			C		C						C	F	F		
812	GL					C										
820	SQDG	18:1	16:0	F	F	C		F	F		F	F		F	F	F
834	SQDG	18:1	17:0	F		C		F	F		F	F	F	F		
846	SQDG	18:1	18:1	F		C		F			F	C		F		F
848	SQDG	18:1	18:0								F	C	C			
850	SQDG	19:0	17:0									C				
860	SQDG	18:1	19:1									C	F			
862	SQDG	19:1	18:0									C	F			
878	Tau	18:1	16:0	F		C			F							
904	Tau	18:1	18:1	F	F	C										

* GL, Glycolipid, either glucosyl- or glucuronosyl-diacyl-glycerol or both (see Table 4 for identification of the compounds); PG, phosphatidyl glycerol; SQDG, sulfoquinovosyl diacylglycerol; Tau, 1,2-diacyl-3- α -D-glucuronopyranosyl-*sn*-glycerol taurineamide.

† F, Ion in negative FAB-MS; C, identified by CID-MS.

Table 6. Sulfolipids found in *Maricaulis* strain VKM B-1513^T

Compound	Mass	Lipid
1	820	1-C18:1-2-C16:0-quinovosyl-glycerol
2	834	1-C18:1-2-C17:0-quinovosyl-glycerol
3	846	1-C18:1-2-C18:1-quinovosyl-glycerol
4	848	1-C18:1-2-C18:0-quinovosyl-glycerol
5	850	1-C19:0-2-C17:0-quinovosyl-glycerol
6	850	1-C18:0-2-C18:0-quinovosyl-glycerol
7	860	1-C18:1-2-C19:1-quinovosyl-glycerol
8	862	1-C19:1-2-C18:0-quinovosyl-glycerol

concentrations above 60 g l⁻¹. Growth of *Maricaulis* strain MCS 26 was reduced somewhat at 5–10 and 80–100 g NaCl l⁻¹, while growth without salt was relatively slow. While tolerance of 100 g NaCl l⁻¹ is shared with *M. maris*, the latter could not grow without salt (Abraham *et al.*, 1999). *Maricaulis* strains VKM 1513^T and VKM 1514 could not grow on PYE medium without NaCl, but optimal growth occurred between 5 and 100 g NaCl l⁻¹.

All *Maricaulis* strains grew best at temperatures between 30 and 40 °C. *Maricaulis* strain VKM B-1513^T grew best between 20 and 40 °C and even showed some growth at 10 °C. Similar behaviour was observed

for *Maricaulis* strain MCS 18^T, although *Maricaulis* strain MCS 6^T grew considerably faster at 10 °C and even showed some growth at 4 °C, at which temperature most of the other *Maricaulis* strains could not grow. *Maricaulis* strain MCS 26 grew best at 40 °C, showed somewhat reduced growth at 30 °C and showed almost no growth at 10 °C.

DISCUSSION

The branching order and evolutionary distances, represented by the branch lengths in Fig. 1, suggested that the sea-water caulobacteria of this study belong to the genus *Maricaulis*. Fig. 1 also revealed that strains described as *Maricaulis* spp. constitute distinct lineages, and the novel species described in this paper comprise two distinct groups that are clearly separate from the type species of *Maricaulis*, *M. maris*. Whereas the evolutionary distances imply the systematic distinction of MCS 6^T and MCS 18^T in one group and VKM B-1513^T, VKM B-1514, MCS 25^T and MCS 26 in the other, the species delineation within these two groups is not so significant by 16S rDNA analysis.

In a previous study (Abraham *et al.*, 1999), *C. halobacteroides* ATCC 15269^T was shown to be synonymous with *Maricaulis* (*Caulobacter*) *maris* ATCC 15268^T, based on lipid and fatty acid content, 16S rDNA analysis, ITS1 patterns, DNA–DNA hybridization and the very small number of observable phenotypic differences. Although the 16S rDNA sequences of ATCC 15269^T and ATCC 15268^T were observed to be identical, this alone would not have justified merging these two species. Sequence comparison of 16S rRNA genes, in general, determines relationships between bacterial genera and is also used widely to resolve intragenetic relationships. However, the genetic standard for species delineation in bacterial systematics is DNA–DNA hybridization (Wayne *et al.*, 1987). Although good correlations have been shown between 16S sequence similarity and DNA–DNA hybridization values (Stackebrandt & Goebel, 1994; Keswani & Whitman, 2001), examples exist where 16S rRNA gene analyses have failed to distinguish sufficiently between species that are clearly separate by phenotypic traits and hybridization values (Hauben *et al.*, 1997). In this study, we observed only a single nucleotide difference between *Maricaulis* strains MCS 6^T and MCS 18^T. Almost-identical 16S rDNA sequences have also been found between species of *Pseudomonas* (Anzai *et al.*, 2000) and, although DNA–DNA hybridization experiments of the respective species pairs have not yet been published, it became apparent from the original publications that significant differences existed in the metabolic traits of these organisms (Elomari *et al.*, 1997; Nishimori *et al.*, 2000).

The same situation apparently exists in the second cluster within *Maricaulis*. The 16S rDNA sequences of strains VKM B-1513^T/VKM B-1514 and MCS 26

differed only in the deletion of one base pair in the stem of helix 6 in the 16S rRNA genes of strains VKM B-1513^T and VKM B-1514. Again, these strains are genetically highly related, and the definition of distinct taxa could only be justified in combination with results obtained from other methods taken together. Strain MCS 25^T could be discriminated genetically from MCS 26 and VKM B-1513^T with greater confidence. The lower 16S rDNA similarity values of strain MCS 25^T to VKM B-1513^T and to MCS 26 were reflected in relatively larger differences in all methods applied in this study. The five marine strains that cluster with *M. maris* ATCC 15268^T (Fig. 1) possessed highly similar 16S rDNA sequences (> 99.2% sequence similarity) but, judging from the results presented in this study, may represent three additional species of *Maricaulis*.

Inasmuch as differences were not observed in the number (a single band was detected for all the strains) and length of the amplified ITS1 (differences in mobility were also not appreciated), we applied a progressive method where higher levels of resolution were achieved. Such a consecutive ARDRA–SSCP identification analysis, applied successfully in this and previous studies (Guasp *et al.*, 2000), permitted us to confirm a clear differentiation, presumably at the species level, in three molecular typing groups. A pattern-based cluster was obtained for strains VKM B-1513^T (= VC-5^T) and VKM B-1514 (= VC-13), which were not differentiated even after SSCP analysis. *Maricaulis* strain MCS 26 was closely related to those strains described above, and this was demonstrated by the fact that the three strains shared the same ARDRA pattern, although they were differentiated after the SSCP analysis, in which band heterogeneities were detected (data not shown). After ARDRA analysis, *Maricaulis* strain MCS 25^T already showed a clearly distinct pattern, suggesting a more distant position from the other strains. Consequently, the results obtained by ITS1 analysis suggest the clear existence of a *Maricaulis* species represented by strains VKM B-1513^T and VKM B-1514, and these results were strongly supported by additional data obtained from the other approaches used in this study. However, although ITS1 analysis suggested a differentiation of strain MCS 26 from strains VKM B-1513^T and VKM B-1514, an even clearer differentiation was obtained of strain MCS 25^T from all the other strains, suggesting the inclusion of this strain in a clearly differentiated ITS1 molecular typing group, i.e. in a distinct species.

The DNA–DNA hybridization value between strains VKM B-1513^T and MCS 26 was 70%, with a rather large error (16%). This value is exactly at the limit where two strains, by definition, belong to the same species. However, strain MCS 26 differed from strain VKM B-1513^T by its composition of polar lipids (reduced number of sulfolipids), its fatty acid composition (less 11:0 iso 3OH), the lack of growth at 20 °C and some growth without NaCl. It can also be differentiated from the other strains by different 16S rRNA gene sequences and in the ITS1 region. All these

differences in largely unrelated properties taken together do not allow an unambiguous inclusion of these strains in the same species. Although DNA–DNA hybridization showed that strains MCS 6^T and MCS 18^T are closely related (59%), they represent distinct species.

The characteristic fatty acids found for the type species of *Maricaulis*, *M. maris*, and related strains were also found in the strains analysed in this study (Abraham *et al.*, 1999). The fatty acids 12:1 3OH and 14:0, typical for strains of *Caulobacter*, were not found in members of this genus, while 15:0 was found only in trace amounts. The fatty acid 12:0 3OH, present in all *Brevundimonas* strains, was found in trace amounts only in *Maricaulis* strain MCS 26. All *Maricaulis* strains contained 11:0 iso 3OH, 17:0 iso, 17:1 iso ω 9c and 18:1 ω 9c. Therefore, the results confirmed that these fatty acids are useful biomarkers to distinguish between these three genera. Within the genus *Maricaulis*, strains MCS 25^T, VKM B-1513^T, VKM B-1514 and MCS 26 differ from all other *Maricaulis* strains by higher content of the fatty acids 18:0, 11-methyl 18:1 ω 5t (ECL 18-080), ECL 18-424 and ECL 18-797 and the lack of 16:1 ω 9c. *Maricaulis* strains MCS 25^T and MCS 26 have the lowest contents of 17:1 iso ω 9c of all *Maricaulis* strains analysed so far. *Maricaulis* strains MCS 6^T and MCS 18^T have the highest content of 17:1 iso ω 9c of all *Maricaulis* strains, but almost no 18:0 or 11:0 iso 3OH. Finally, strains VKM B-1513^T and VKM B-1514 have large amounts of 11:0 iso 3OH. Considering the fact that the 16S rRNA gene sequences of the *Maricaulis* species are very similar (> 99% similarity), the fatty acids of the total lipids proved to be a powerful tool for the discrimination of the distinct *Maricaulis* species.

The tendency of caulobacteria to have large amounts of glycolipid was corroborated in this study, and a multitude of different glycolipids was identified. NMR and MS of the polar lipid fraction revealed that all glycolipids belong to the α -glucosyl and α -glucuronosyl diacyl glycerol types. The latter is the more abundant and diverse of the two. Surprisingly, phosphoglycerols are rare and found only in *M. maris* and the closely related strain MCS 28. In *Maricaulis* strains, phospholipids are mainly replaced by sulfolipids, which are the main polar lipids in *Maricaulis* strain VKM B-1513^T. In contrast to *M. maris*, phospholipids were not found in any of the novel *Maricaulis* species, and taurineamides were also absent. Instead of these lipids, sulfolipids based on glucose, like the glycolipids, and belonging to the SQDG type, were detected. The largest amounts and the greatest variety of these lipids were elucidated in *Maricaulis* strains VKM B-1513^T and VKM B-1514. This species is remarkable in this respect compared with all other *Maricaulis* species. A number of these sulfolipids are described here for the first time. This makes this species a much better producer of these compounds than various cyanobacteria, which have been used to produce such lipids possessing anti-HIV-

1 (Gustafson *et al.*, 1989) and anti-tumour-promoter activities (Shirahashi *et al.*, 1993).

Conclusions

Due to the heterogeneity of the isolates of *Maricaulis*, distinct taxa can be discerned and the proposal of four novel species of *Maricaulis* is justified.

Description of *Maricaulis parjimensis* sp. nov.

Maricaulis parjimensis (par.ji.men'sis. N.L. adj. *parjimensis* of Parjim, the capital of the Indian state of Goa, referring to the isolation of the type strain from the nearby coast).

Can grow on PYE medium with 5–100 g NaCl l⁻¹, with optimal growth between 20 and 80 g NaCl l⁻¹. No growth is observed without NaCl; slow growth is also found with 100 g NaCl l⁻¹. Optimal growth temperature is 30–40 °C; does not grow at 10 or 50 °C. Characterized by two major fatty acids, 18:0 and 18:1 ω 7; minor fatty acids are 17:0, 17:1 ω 8c and 18:1 ω 9c. Polar lipids are α -D-glucopyranosyl diacylglycerol, α -D-glucuronopyranosyl diacylglycerol and SQDG, but no phosphatidyl diacylglycerol or α -D-glucuronopyranosyl diacylglycerol taurineamide. Isolated from sea water off the coast of Goa, India. The G+C content of the type strain is 63.0 mol%. The type strain is strain MCS 25^T (= LMG 19863^T = CIP 107440^T).

Description of *Maricaulis salignorans* sp. nov.

Maricaulis salignorans (sa.lig.nor'ans. L. n. *sal* salt; L. adj. *ignorans* ignoring; N.L. adj. *salignorans* salt-ignoring, referring to the ability to grow without added salt).

The description is the same as that given for the genus (Abraham *et al.*, 1999), with the following additional characteristics. Can grow on PYE medium with 0–80 g NaCl l⁻¹, with optimal growth between 20 and 60 g NaCl l⁻¹. Although NaCl is required for optimal growth, only slightly reduced growth is observed without NaCl. This fairly rapid growth in freshwater is unusual for this genus and is distinct from *M. maris* (synonym *C. halobacteroides*) and *Maricaulis* strain MCS 26. Strains do not tolerate salt concentrations above 80 g NaCl l⁻¹. Characterized by two major fatty acids, 16:0 and 17:1 iso ω 9c; minor fatty acids are 14:1 ω 5, 17:1 ω 8c, iso-17:0 and 17:0. Polar lipids are α -D-glucopyranosyl diacylglycerol, α -D-glucuronopyranosyl diacylglycerol and SQDG, but no phosphatidyl diacylglycerol or α -D-glucuronopyranosyl diacylglycerol taurineamide. The G+C content of the type strain is 63.3 mol%. Isolated from sea water off Salsbury Point County Park, WA, USA. The type strain is strain MCS 18^T (= LMG 19864^T = CIP 107439^T).

Description of *Maricaulis washingtonensis* sp. nov.

Maricaulis washingtonensis (wa.shing.ton.en'sis. N.L. adj. *washingtonensis* of the US state of Washington, referring to the location of isolation of the type strain).

The description is the same as those given for the genus (Abraham *et al.*, 1999) and for *M. salignorans*, with the following additional characteristics. Can grow on PYE medium with 0–80 g NaCl l⁻¹, with optimal growth between 20 and 40 g NaCl l⁻¹. Although NaCl is required for optimal growth, only slightly reduced growth is observed without NaCl. At NaCl concentrations above 40 g l⁻¹, a long decline of the growth rate is observed until, for 100 g NaCl l⁻¹, no growth is observed. The G+C content of the type strain is 63.0 mol %. A DNA–DNA binding value of 59 % was found between *M. washingtonensis* MCS 6^T and *M. salignorans* MCS 18^T. Isolated from sea water from the Inner Marina, Edmonds, WA, USA. The type strain is strain MCS 6^T (= LMG 19865^T = CIP 107441^T).

Description of *Maricaulis virginensis* sp. nov.

Maricaulis virginensis (vir.gin.en'sis. N.L. adj. *virginensis* from the Virgin Islands).

The description is the same as that given for the genus (Abraham *et al.*, 1999), with the following additional characteristics. All strains are characterized by two major fatty acids, 17:0 and 18:1 ω 7; minor fatty acids are 14:0, 16:0, 17:0 iso and 18:0. The main polar lipids are α -sulfoquinovosyl diacylglycerols. Cannot grow on PYE medium without NaCl, and optimal growth occurs between 5 and 100 g NaCl l⁻¹. Grows at temperatures between 10 and 40 °C, with optimal growth at 20–40 °C. A DNA–DNA binding value of 83 % was found between *M. virginensis* strains VKM B-1513^T and VKM B-1514. A DNA–DNA binding value of 70 % was found between *M. virginensis* VKM B-1513^T and strain MCS 26 (= LMG 19862). This is just at the margin to include this strain in this taxon; however, strain MCS 26 displays a number of differences from *M. virginensis*; therefore, it is not included here in this species. The type strain was obtained from deep sea water near a hydrothermal vent off the Virgin Islands. The G+C content of the type strain is 65.2 mol %. The type strain is strain VKM B-1513^T (= LMG 21018^T = VC-5^T = CIP 107438^T). Strain VC-13 (= VKM B-1514) is a closely related strain.

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