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Phylogeny and polyphasic taxonomy of *Caulobacter* species. Proposal of *Maricaulis* gen. nov. with *Maricaulis maris* (Poindexter) comb. nov. as the type species, and emended description of the genera *Brevundimonas* and *Caulobacter*

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The genus *Caulobacter* is composed of prosthecate bacteria often specialized for oligotrophic environments. The taxonomy of *Caulobacter* has relied primarily upon morphological criteria: a strain that visually appeared to be a member of the *Caulobacter* has generally been called one without challenge. A polyphasic approach, comprising 16S rDNA sequencing, profiling restriction fragments of 16S–23S rDNA interspacer regions, lipid analysis, immunological profiling and salt tolerance characterizations, was used to clarify the taxonomy of 76 strains of the genera *Caulobacter*, *Brevundimonas, Hyphomonas* and *Mycoplana*. The described species of the genus *Caulobacter* formed a paraphyletic group with *Caulobacter henricii, Caulobacter fusiformis, Caulobacter vibrioides* and *Mycoplana segnis* (*Caulobacter segnis* comb. nov.) belonging to *Caulobacter sensu stricto. Caulobacter bacteroides* (*Brevundimonas bacteroides* comb. nov.), *C. henricii* subsp. *aurantiacus* (*Brevundimonas aurantiaca* comb. nov.), *Caulobacter intermedius* (*Brevundimonas intermedia* comb. nov.), *Caulobacter subvibrioides* (*Brevundimonas subvibrioides* comb. nov.), *C. subvibrioides* subsp. *albus* (*Brevundimonas alba* comb. nov.), *Caulobacter variabilis* (*Brevundimonas variabilis* comb. nov.) and *Mycoplana bullata* belong to the genus *Brevundimonas*. The halophilic species *Caulobacter maris* and *Caulobacter halobacteroides* are different from these two genera and form the genus *Maricaulis* gen. nov. with *Maricaulis maris* as the type species. *Caulobacter leidyia* was observed to cluster with species of the genus *Sphingomonas. Caulobacter crescentus* is synonymous with *C. vibrioides* and *C. halobacteroides* is synonymous with *Maricaulis maris* as determined by these analyses and DNA–DNA hybridization. Biomarkers discerning these different genera were determined. The necessary recombinations have been proposed and a description of *Maricaulis* is presented.

Keywords: *Caulobacter*, *Brevundimonas*, *Maricaulis* gen. nov., *Mycoplana*, *Hyphomonas*

Abbreviations: DCI, desorption chemical ionization; FAB, fast atom bombardment; ISR, interspacer region; NFM, non-fat dry milk; SSCP, single-strand conformational polymorphism.

The EMBL accession numbers for the 16S rDNA sequences reported in this paper are listed in Table 1.

INTRODUCTION

The genus *Caulobacter* is composed of dimorphic, prosthecate bacteria. In these prokaryotes, reproduction results in the separation of two cells that are morphologically and behaviourally different from each other. One sibling is non-motile, sessile by virtue of adhesive material and prosthecate, possessing at least one elongated, cylindrical appendage (a prostheca) that is an outgrowth of the cell envelope, including the outer membrane, the peptidoglycan layer and the cell membrane (Staley, 1968). The other sibling 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 reproductive cycle. It is regarded as a reflection of an ecological programme helping to disperse the population at each generation, thereby minimizing competition for resources.

In each species studied so far, the motile cell grows less actively than its prosthecate sibling. Progress toward reproduction is initiated in the swarmers only after this period of motility. It is consistent with this developmental and reproductive habit that these bacteria exhibit the physiological properties of oligotrophs (Poindexter, 1981a, b) and, most importantly, the tolerance of prolonged nutrient scarcity. Once isolated, individual strains may tolerate much higher nutrient concentrations than are useful for their enrichment and isolation. All caulobacteria are oxybiotic and grow in well-aerated cultures.

Caulobacteria are ubiquitous in water. As typical aquatic bacteria, they may be second only to pseudomonads in the breadth of their distribution and numbers (Lapteva, 1987). These two bacterial groups together are presumed to be responsible for considerable mineralization of dissolved organic material in aquatic environments, whereby the oligotrophic caulobacters are especially important when nutrient concentrations and ambient temperatures are low (Staley *et al*., 1987). Practically any type of seawater contains *Caulobacter* (Jannasch & Jones, 1960; Anast & Smit, 1988) and, further, MacRae & Smit (1991) reported the ready detection of *Caulobacter* in highly aerobic activated sewage sludge.

The first isolation of a *Caulobacter* sp. was reported by Loeffler (1890). He noticed the unusually low nutrient requirements of the strain. He could not assign the strain to a known taxon and named this 'höchst merkwürdigen Organismus' '*Vibrio* (*?*) *spermatozoides*'. Later, Henrici & Johnson (1935) described the genus *Caulobacter* with *Caulobacter vibrioides* as the type species. Then, Poindexter (1964) enlarged the monotypic genus considerably by describing nine species and two subspecies, and later added *Caulobacter variabilis* (Poindexter, 1989).

Henrici & Johnson (1935) did not isolate *Caulobacter vibrioides* and therefore a type strain was not named. Poindexter (1964) proposed strain CB51 as the neotype strain of *C. vibrioides* (Poindexter & Lewis, 1966). Strain CB51^T was obtained independently from the All-Russian Collection of Microorganisms (VKM B-1496^T) and from J. T. Staley (= DSM 9893^T).

Fatty acids of *Caulobacter* spp. were determined by Carter & Schmidt (1976) and polar lipids have been analysed by various groups (Minnikin *et al*., 1973; Wilkinson, 1969; Wilkinson & Bell, 1971; Andreev *et al*., 1986; Batrakov *et al*., 1996, 1997). Using 5S rRNA sequences, Stackebrandt *et al*. (1988) determined the phylogenetic position of *Caulobacter crescentus* within the α-subclass of the *Proteobacteria*; similar results were reported two years later by Nikitin *et al*. (1990). Anast & Smit (1988) were the first to determine differences between freshwater and marine caulobacteria on a large set of strains. In a pioneering study, Stahl *et al*. (1992) sequenced the 16S rRNA of several *Caulobacter* isolates. Although sequences of type strains of *Caulobacter* species were not included in this study and a strain wrongly labelled as *Caulobacter subvibrioides* was included (Sly *et al*., 1997), this study revealed, for the first time, that *Caulobacter* actually forms two different lineages, one comprising the freshwater and brackish water *Caulobacter* spp. and the other comprising the marine forms. Stahl *et al*. (1992) also showed that *Brevundimonas diminuta* (Segers *et al*., 1994) clusters within the group of freshwater *Caulobacter* spp. The 16S rDNA sequences of *Mycoplana segnis* and *Mycoplana bullata* (Yanagi & Yamasato, 1993) also fall in the freshwater *Caulobacter*/*Brevundimonas* cluster and thus complicate the taxonomy further.

As caulobacteria are ubiquitous in water samples and are believed to play an important role in the carbon cycling within their habitats, the intent of the present study was to evaluate the diversity of freshwater and marine caulobacters using lipid analysis, immunological profiling and 16S rRNA gene sequences to compare these results with physiological data. Because previous studies (Stahl *et al*., 1992) revealed a close phylogenetic distance between *Caulobacter*, *Hyphomonas jannaschiana*, *Mycoplana bullata*, *Mycoplana segnis* and *B. diminuta*, strains of these genera were also included in this study.

METHODS

Strains and culture conditions. The strains in this study were obtained from the American Type Culture Collection (ATCC), the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; DSM strains), the Laboratorium voor Microbiologie, Universiteit Gent, Belgium (LMG strains), the All-Russian Collection of Microorganisms (VKM), from Ingo Fritz at the GBF (*Brevundimonas* sp. BO.07 and BO.10) and from the Dept of Microbiology and Immunology at the University of British Columbia (CB, FWC and MCS strains). Table 1 shows the origin of the isolates.

All strains were grown in medium PYEM with the exception of *Caulobacter maris* ATCC 15268^T, *Caulobacter halobacteroides* ATCC 15269^T, *Hyphomonas* spp. and all *Caulo-*

Table 1. List of strains used in this study including their serum reactions and the accession numbers of the newly determined 16S rRNA gene sequences

Strain	Source	Positive reaction with serum:	Accession no.
Brevundimonas diminuta LMG 2089^T	Freshwater	SMT2	AJ227778
Brevundimonas diminuta LMG 2337	Blood, patient with endocarditis	SMT2	AJ227779
Brevundimonas sp. BO.07	Marine isolate, sampling depth 110 m, offshore Nice/Mediterranean Sea	SMT2	AJ227800
Brevundimonas sp. BO.10	Marine isolate, sampling depth 150 m, offshore Nice/Mediterranean Sea	SMT2	AJ227801
Brevundimonas vesicularis LMG 2350^T	Leech (*Hirudo* sp.), urinary bladder, epithelium	SMT2	AJ227780
Brevundimonas vesicularis LMG 11141	Pleural liquid, Hospital, Brussels, Belgium	SMT2	AJ227781
Caulobacter bacteroides LMG 15096^T	Freshwater lake	SMT2	AJ227782
Caulobacter crescentus CB15	Pond water	SMT7	AJ227757
Caulobacter crescentus CB2^T	Tap water	SMT7	AJ227756
Caulobacter fusiformis ATCC 15257^T	Pond water	SMT7	AJ227759
Caulobacter halobacteroides ATCC 15269^T	Filtered seawater	SMT25	AJ227803
Caulobacter henricii ATCC 15253^T	Freshwater pond	SMT7	AJ227758
Caulobacter henricii subsp. *aurantiacus* DSM 4731	Contaminated *Chlorella* culture, CB-R^T	SMT2	AJ227787
Caulobacter intermedius ATCC 15262^T	Pond water	SMT2	AJ227786
Caulobacter leidyia ATCC 15260^T	Millipede hind gut	NR	AJ227812
Caulobacter leidyia DSM 4733^T		NR	
Caulobacter maris ATCC 15268^T	Filtered seawater	SMT25	AJ227802
Caulobacter sp. DSM 6811 (= ATCC 15256)		NT	AJ227789
Caulobacter sp. DSM 6812 (= ATCC 15267)	Pond and tap water mixture	NT	AJ227790
Caulobacter sp. DSM 10556 (= ATCC 15263)	Pond water	NT	AJ227788
Caulobacter sp. DSM 10560 (= ATCC 15259)	Pond water	NT	AJ227791
Caulobacter sp. DSM 10670 (= ATCC 15258)	Pond water	NT	AJ227792
Caulobacter sp. FWC 2	Lake Washington, Seattle, WA, USA	SMT7	AJ227760
Caulobacter sp. FWC 4	Freshwater slough adjacent to Lake Washington, Seattle, WA, USA	SMT2	AJ227793
Caulobacter sp. FWC 5	Surface water in waterlogged area, Bothell, WA, USA	SMT7	AJ227794
Caulobacter sp. FWC 6	Tap water, Oakland, CA, USA	SMT7	AJ227761
Caulobacter sp. FWC 7	Tap water, Oakland, CA, USA	SMT7	
Caulobacter sp. FWC 8	Secondary treatment, activated sludge process, Bio-P, Univ. British Columbia, BC, Canada	SMT7	AJ227762
Caulobacter sp. FWC 9	Bench-scale secondary treatment system, fed with landfill leachate, Univ. British Columbia, BC, Canada	SMT7	
Caulobacter sp. FWC 11	Holding tank for raw influent sewage, Univ. British Columbia, BC, Canada	SMT7	
Caulobacter sp. FWC 12	Bench-scale secondary treatment system, fed with landfill leachate, Univ. British Columbia, BC, Canada	SMT7	
Caulobacter sp. FWC 13	Surface water from a peat bog, Richmond, BC, Canada	SMT7	
Caulobacter sp. FWC 14	Secondary treatment, activated sludge process, Univ. British Columbia, BC, Canada	SMT2	AJ227795
Caulobacter sp. FWC 15	Primary treatment facility for Greater Vancouver, Iona Island, BC, Canada	SMT7	
Caulobacter sp. FWC 16	Primary treatment facility for Greater Vancouver, Iona Island, BC, Canada	SMT7	AJ227763

Table 1 (*cont.*)

Strain	Source	Positive reaction with serum:	Accession no.
Caulobacter sp. FWC 17	Secondary treatment facility, rotating biological contactor, Langley, BC, Canada	SMT7	AJ227764
Caulobacter sp. FWC 18	Primary treatment facility for Greater Vancouver, Iona Island, BC, Canada	SMT7	AJ227765
Caulobacter sp. FWC 19	Secondary treatment, activated sludge process, Univ. British Columbia, BC, Canada	SMT7	
Caulobacter sp. FWC 20	Secondary treatment, activated sludge process, Univ. British Columbia, BC, Canada	SMT7	AJ227766
Caulobacter sp. FWC 21	Bench-scale rotating biological contactor, Univ. British Columbia, BC, Canada	SMT7	AJ227767
Caulobacter sp. FWC 22	Secondary treatment facility, aerobic digester, Langley, BC, Canada	SMT7	
Caulobacter sp. FWC 25	Secondary treatment facility, primary treatment region, Edmonton, Alberta, Canada	SMT7	
Caulobacter sp. FWC 26	Secondary treatment facility, activated sludge, Edmonton, Alberta, Canada	SMT7	AJ227768
Caulobacter sp. FWC 27	Contour trench wastewater treatment system, Takla, BC, Canada	SMT7	
Caulobacter sp. FWC 28	Contour trench wastewater treatment system, Takla, BC, Canada	SMT7	AJ227769
Caulobacter sp. FWC 29	Secondary treatment facility, activated sludge system, Bozeman, MT, USA	SMT7	AJ227770
Caulobacter sp. FWC 30	Untreated influent sewage, Gold Bar facility, Edmonton, Alberta, Canada	SMT2	AJ227796
Caulobacter sp. FWC 31	Contour trench wastewater treatment system, Takla, BC, Canada	SMT7	AJ227771
Caulobacter sp. FWC 32	Trickle filter secondary treatment facility, Coeur d'Alene, ID, USA	SMT7	
Caulobacter sp. FWC 33	Trickle filter secondary treatment facility, Coeur d'Alene, ID, USA	SMT7	AJ227772
Caulobacter sp. FWC 34	Secondary treatment facility, activated sludge system, Pullman, WA, USA	SMT7	
Caulobacter sp. FWC 35	Trickle filter secondary treatment facility, Las Vegas, NV, USA	SMT7	AJ227773
Caulobacter sp. FWC 37	Secondary treatment facility, activated sludge system	SMT7	
Caulobacter sp. FWC 38	Secondary treatment facility, activated sludge system	SMT7	AJ227774
Caulobacter sp. FWC 39	Secondary treatment facility, activated sludge, Kelowna, BC, Canada	SMT7	
Caulobacter sp. FWC 40	Secondary treatment facility, activated sludge, Kelowna, BC, Canada	SMT2	AJ227797
Caulobacter sp. FWC 41	Secondary treatment facility, activated sludge, Calgary, Alberta, Canada	SMT7	AJ227775
Caulobacter sp. FWC 42	Secondary treatment facility, activated sludge, Calgary, Alberta, Canada	SMT7	AJ227776
Caulobacter sp. FWC 43	Secondary treatment facility, activated sludge, Calgary, Alberta, Canada	SMT2	AJ227798
Caulobacter sp. FWC 44	Activated sludge, secondary sewage treatment plant, Univ. British Columbia, BC, Canada	SMT7	

Table 1 (*cont.*)

Strain	Source	Positive reaction with serum:	Accession no.
Caulobacter sp. FWC 45	Stream water, Burnaby, BC, Canada	SMT7	AJ227777
Caulobacter sp. MCS 6	Seawater, Inner marina, Edmonds, WA, USA	NT	AJ227804
Caulobacter sp. MCS 10	Filtered seawater, Anacortes, WA, USA	SMT25	
Caulobacter sp. MCS 11	Filtered seawater, Anacortes, WA, USA	NT	AJ227805
Caulobacter sp. MCS 17	Brackish water slough adjacent to Arness Park, Kingston, WA, USA	SMT2	AJ227799
Caulobacter sp. MCS 18	Seawater, Salsbury Point County Park, Adjacent to Hood Canal Bridge, WA, USA	SMT25	AJ227806
Caulobacter sp. MCS 23	Filtered seawater, Univ. California, Santa Barbara Marine Laboratory, CA, USA	NT	AJ227807
Caulobacter sp. MCS 24	Brackish water; creek flowing into saltwater at Carkeek Park, Seattle, WA, USA	SMT2	
Caulobacter sp. MCS 25	Littoral sample from Indian Ocean, Goa, India	SMT25	AJ227808
Caulobacter sp. MCS 26	Littoral sample from Indian Ocean, Goa, India	SMT25	AJ227809
Caulobacter sp. MCS 28	Filtered seawater, Burrard Inlet, Vancouver, BC, Canada	SMT25	AJ227810
Caulobacter sp. MCS 33	Above the hot water plume of a deep-sea hydrothermal vent, near Vancouver Island, Canada	NT	AJ227811
Caulobacter subvibrioides LMG 14903^T	Pond water	SMT2	AJ227784
Caulobacter subvibrioides subsp. *albus* DSM 4736^T	Soil, CB88^T	SMT2	AJ227785
Caulobacter variabilis ATCC 15255^T	Pond water	SMT2	AJ227783
Caulobacter vibrioides ATCC 11764		SMT7	AJ227755
Caulobacter vibrioides DSM 9893^T		SMT7	AJ227754
Caulobacter vibrioides VKM B-1496^T		NT	AJ009957
Hyphomonas jannaschiana ATCC 33883^T	Mussel bed in Galapagos thermal vent	NT	AJ227814
Hyphomonas polymorpha DSM 2665^T	Human nasal sinus infection	NT	AJ227813
Mycoplana bullata DSM 7126^T	Soil	SMT2	
Mycoplana segnis DSM 7131^T	Soil	NT	

NR, No reaction with sera SMT2, SMT7 or SMT25; NT, not tested.

bacter sp. MCS strains, which were grown in the marine medium SPYEM. Freshwater *Caulobacter* medium PYEM contained 2 g peptone, 2 g yeast extract, 0·5 g NH_4Cl and 1 l deionized water. After autoclaving and cooling, 5 ml riboflavin (0·2 mg ml^{-1}, filter-sterilized), 2 ml 50 % glucose (sterile), 1 ml 20 % $MgSO_4$ (sterile) and 1 ml 10 % $CaCl_2$ (sterile) were added. Marine *Caulobacter* medium SPYEM contained 30 g sea salts (Sigma), 0·5 g NH_4Cl and 1 l deionized water. After autoclaving and cooling, 20 ml 50 × PYE [50 × PYE: 100 g peptone and 50 g yeast extract in 1 l deionized water (autoclaved)], 2 ml 50 % glucose and 5 ml riboflavin (0·2 mg ml^{-1}) were added. The strains were incubated in 2 l flasks at 30 °C, shaken at 100 r.p.m., and the biomass was harvested by centrifugation at room temperature and 5860 ***g*** in the late exponential phase after 72 h.

DNA isolation and base composition. High molecular mass genomic DNA was isolated and purified with the blood and cell culture DNA kit and Genomic-tips (Qiagen) according to the manufacturer's protocols. DNA was enzymically digested using nuclease P1 and bacterial alkaline phosphatase, and the mean G+C content was determined by HPLC (Tamaoka & Komagata, 1984); G+C contents were calculated according to Mesbah *et al.* (1989) using non-methylated lambda phage DNA (Sigma) as a standard.

16S rDNA sequencing and analysis. Single colonies were picked from agar, suspended in 100 µl TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8·0) and boiled for 5 min. The lysate was centrifuged briefly and 1 µl supernatant was used for PCR, targeting the 16S rRNA genes (Medlin *et al.*, 1988; Edwards *et al.*, 1989) using a forward primer, hybridizing at positions 8–27, and a reverse primer, hybridizing at the complement of positions 1525–1541 (*Escherichia coli* 16S rRNA gene sequence numbering). PCR was carried out using conditions described previously (Karlson *et al.*, 1993). Amplified 16S rDNA was purified using Microcon 100 microconcentrators (Amicon) and the sequences were determined directly using an Applied Biosystems 373A DNA sequencer and the protocols recommended by the manufacturer (Perkin Elmer) for *Taq*-polymerase-initiated cycle

sequencing with fluorescent-dye-labelled dideoxynucleotides. The 16S rRNA sequencing primers have been described previously (Lane, 1991). The nucleotide sequences were aligned with reference 16S rRNA and 16S rRNA gene sequences using evolutionary conserved primary sequence and secondary structure (Gutell *et al*., 1985) as references. Evolutionary distances (Jukes & Cantor, 1969) were calculated from nearly complete sequence-pair dissimilarities using only homologous, unambiguously determined nucleotide positions. Phylogenetic trees were constructed using the software programs of the PHYLIP package (Felsenstein, 1989).

16S–23S rDNA interspacer based analysis. DNA extractions and PCR were performed as described for 16S rDNA sequencing with the following differences. The forward primer was 16f945, corresponding to positions 927–945 of the *E. coli* 16S rDNA (Brosius *et al*., 1978) and the reverse primer was 23r458, corresponding to positions 458–473 of the *E. coli* 23S rDNA (Brosius *et al*., 1980). The interspacer region (ISR) PCR products were digested using the tetrameric endonuclease *Taq*I (Boehringer Mannheim) at 65 °C, according to the supplier's recommendations. The restriction fragment products were separated by agarose (3 %, w/v) gel electrophoresis, stained with ethidium bromide and visualized by UV excitation. For single-stranded conformational polymorphism (SSCP) analysis of the restriction fragments (Orita *et al*., 1989), 5 µl digested ISR PCR products was diluted 1:1 in denaturing solution (99 % deionized formamide, 0·05 % xylene cyanol and 0·04 % bromophenol blue), incubated at 95 °C for 5 min and placed directly into ice water to prevent reannealing of the single-stranded product. The products were electrophoresed in non-denaturing polyacrylamide precasted gels (CleanGel DNA Analysis kit; Pharmacia Biotech) on a Multiphor II Electrophoresis unit at 200 V for 10 min followed by 45 min at 600 V. After electrophoresis, the gel was stained with the Plus One silver staining kit (Pharmacia Biotech) following the protocol of the manufacturer.

DNA–DNA hybridization. Chromosomal DNA of high molecular mass was isolated according to the method of Marmur (1961). Degrees of DNA–DNA binding, expressed as percentages, were determined spectrophotometrically using the initial renaturation method of De Ley *et al.* (1970) and the equipment and method described by Willems *et al.* (1989).

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/*tert*-butyl methyl ether (1:1, v/v)] as described in detail by Osterhout *et al.* (1991). Fatty acid methyl esters were analysed by GC. Separation of fatty acid methyl esters was achieved using fused-silica capillary column (25 m × 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 *et al.* (1991). The instrument was equipped with a flame-ionization detector and an autosampler. H_2 served as the 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).

Desorption chemical ionization (DCI) mass spectrometry. The DCI spectra were performed in positive mode with a double focussing mass spectrometer (Finnigan MAT-95) with an acceleration voltage of 5 kV and a resolution of 1:2300. The ionization was carried out using ammonia as the reactant gas with a source pressure of 10^{-4} mbar, 120 eV ionization energy and a source temperature of 100 °C. The single scans were acquired by scanning the magnet from m/z 100 to 1000 with a scan speed of 1·5 s (mass decade)$^{-1}$ and a heating rate of 488 °C min^{-1} using an ICIS data system for recording the analysis.

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) was used in the negative mode.

^{1}H-NMR spectra were recorded in 7:3 d-chloroform/d_3-methanol at 300 K on a Bruker ARX-400 NMR spectrometer relative to internal tetramethylsilane.

Respiratory lipoquinone analysis. Strains used for respiratory lipoquinone analysis were *B. diminuta* DSM 7234^T, *Brevundimonas vesicularis* DSM 7226^T, *Caulobacter bacteroides* DSM 4726^T (= ATCC 15254^T), *C. crescentus* DSM 4727^T (= ATCC 15252^T), *Caulobacter fusiformis* DSM 4728^T (= ATCC 15257^T), *C. halobacteroides* DSM 4729^T (= ATCC 15269^T), *Caulobacter henricii* DSM 4730^T (= ATCC 15253^T), *C. henricii* DSM 4731 (= ATCC 15266), *Caulobacter intermedius* DSM 4732^T (= ATCC 15262^T), *Caulobacter leidyia* DSM 4733^T (= ATCC 15260^T), *C. maris* DSM 4734^T (= ATCC 15268^T), *C. subvibrioides* DSM 4735^T (= ATCC 15264^T), *C. subvibrioides* DSM 4736 (= ATCC 15265), *C. variabilis* DSM 4737^T (= ATCC 15255), *C. vibrioides* DSM 9893^T (= CB51^T; from J. T. Staley), *C. vibrioides* VKM B-1496^T (= CB51^T; from M. Vainhstein), *C. vibrioides* DSM 4738 (= ATCC 11764), *Caulobacter* sp. DSM 6811 (= ATCC 15256), *Caulobacter* sp. DSM 6812 (= ATCC 15267), *Caulobacter* sp. DSM 10556 (= ATCC 15263), *Caulobacter* sp. DSM 10560 (= ATCC 15259), *Caulobacter* sp. DSM 10670 (= ATCC 15258), *Mycoplana bullata* DSM 7126^T and *Mycoplana segnis* DSM 7131^T.

Respiratory lipoquinones were extracted from 100 mg freeze-dried cell material using the method described by Tindall (1990a, b), in which methanol:hexane was used for extraction and recovery of the respiratory lipoquinones. Respiratory lipoquinones were separated into their different classes (menaquinones and ubiquinones) by TLC on silica gel (Macherey-Nagel), using hexane:*tert*-butyl methyl ether (9:1, v/v) as solvent. UV-absorbing bands corresponding to menaquinones or ubiquinones were removed from the plate and further analysed by HPLC. This step was carried out on an LDC Analytical (Thermo Separation Products) HPLC fitted with a reverse phase column (Macherey-Nagel; 2 × 125 mm, 3 µm, RP18) using methanol as the eluant. Respiratory lipoquinones were detected at 269 nm.

Phenotypic characterization. Type strains and selected isolates were grown in 20 ml PYEM medium amended with 0, 5, 10, 20, 30, 40, 60, 80 or 100 g NaCl l^{-1}. The OD_{600} of the cell suspension was set to 0·3 at the beginning of the experiment and determined at time intervals over 2 d. The differences between these measurements were used to determine salt tolerances.

Preparation of whole-cell lysates and the 43 kDa protein. The protocol for the outer-membrane protein purification (Filip *et al*., 1973; Judd, 1988) was used with modifications as follows. A 50 ml culture of bacteria was grown to an

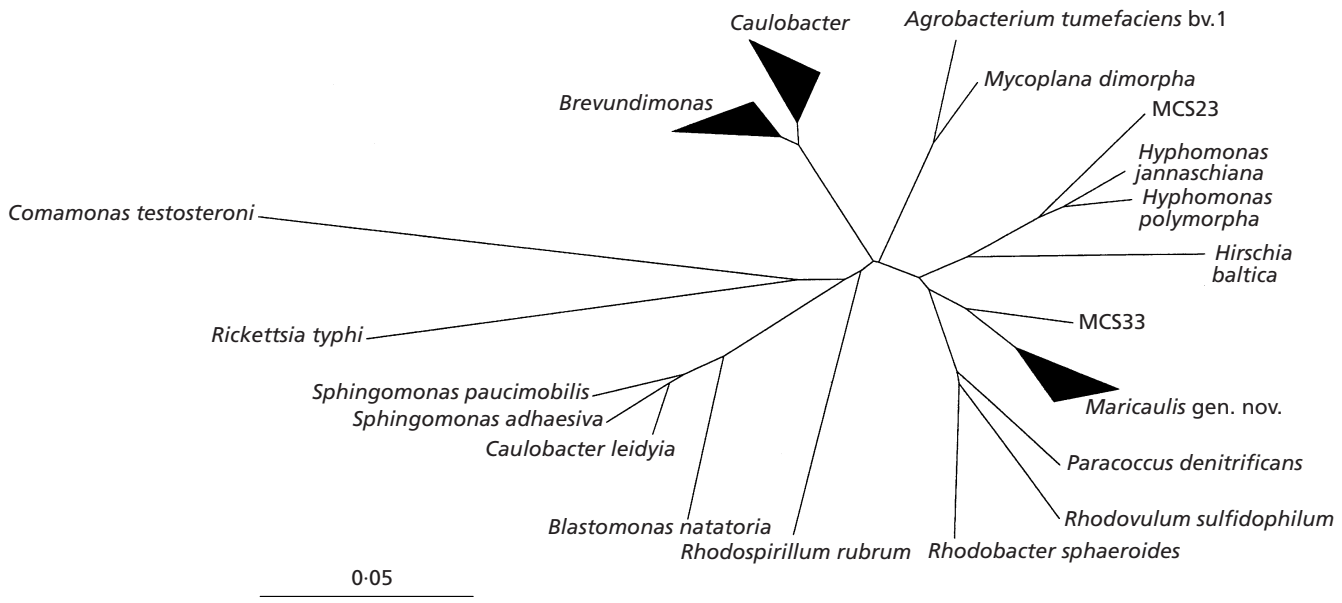


Fig. 1. Unrooted dendrogram based on a comparison of 16S rDNA sequences of strains belonging to *Caulobacter sensu lato, Brevundimonas, Hyphomonas* and *Mycoplana* within the context of the α-lineage of the *Proteobacteria*. The cluster length is depicted as the estimated length of the longest branch within each cluster. Sequence data for strains of other genera were obtained from the GenBank/EMBL and/or RDP databases under the following accession numbers: *Rickettsia typhi* str. Wilmington (L36221), *Blastomonas natatoria* ATCC 35951^T (X73043), *Sphingomonas paucimobilis* DSM 1098^T (X72722), *Sphingomonas adhaesiva* JCM 7370^T (X72720), *Rhodospirillum rubrum* str. ATH 1.1.1. (RDP), *Agrobacterium tumefaciens* bv.1 NCPPB2437 (D14500), *Hirschia baltica* IFAM 1418^T (X52909), *Rhodobacter sphaeroides* IL106 (D16424), *Paracoccus denitrificans* LMG 4218^T (X69159), *Mycoplana dimorpha* ATCC 4279^T (D12786), *Mycoplana bullata* IAM 13153^T (D12785), *Mycoplana segnis* IFO 13240^T (D13947). *Comamonas testosteroni* (RDP) was included as an outgroup. Scale bar, 5 nucleotide substitution per 100 bases.

OD_{600} of 1·0 and washed three times in PBS (137 mM NaCl, 2·7 mM KCl, 8 mM Na_2HPO_4, 1·4 mM KH_2PO_4 adjusted to pH 7·4) and the resulting pellet was resuspended in 4·5 ml buffer A (10 mM Tris/HCl, pH 7·5; 5 mM $MgCl_2$). The cells were disrupted twice in a French press according to the supplier's instructions and cell debris was removed by a short low-speed centrifugation (2000 ***g***). To prepare outer membranes, the whole cell lysate was centrifuged at 15000 ***g*** for 30 min at 4 °C. The pellet was resuspended in 0·5 ml buffer A and sodium lauroyl sarcosinate (Sarkosyl; Serva) added to a final concentration of 2%. Incubation was carried out for 20 min at 20 °C, followed by another centrifugation step at 15000 ***g*** for 30 min at 20 °C. The supernatant fluid was discarded and the pellet was dissolved in double-concentration sample buffer. Samples were subjected to analytical SDS-PAGE [12% (w/v) acrylamide] according to a standard protocol (Maizel, 1969).

Immunoblotting. Bacterial cells (1 ml) with an OD_{600} of 0·8 were harvested by centrifugation at 6000 ***g*** for 5 min at 4 °C and dissolved in 100 µl double-concentration SDS-PAGE sample buffer (Laemmli, 1970), boiled and subjected to SDS-PAGE (12·5%). To avoid different mobilities of proteins due to sensitivity to 2-mercaptoethanol and heat (Hancock & Carey, 1979), samples were boiled for 5 min before electrophoresis. The resulting protein profiles were highly reproducible. Immunoblots were carried out as described previously (Towbin *et al.*, 1979; Tesar & Marquardt, 1990). Nitrocellulose membranes were blocked in 5% non-fat dry milk (NFM) for 1 h at room temperature followed by incubation (1 h) with rabbit immune serum, diluted 1:500 in PBS and supplemented with 2·5% NFM. After washing the membranes three times in PBST (PBS containing 0·05% Tween) for 30 min, peroxidase-conjugated goat anti-rabbit antibodies (1:1500; Dianova) in PBST were added and incubation of the membrane continued for 1 h at room temperature. Final washing was carried out with PBST followed by short incubation in PBS. Blots were developed by the addition of 2·2 mM 4-chloro-1-naphthol and 0·03% (v/v) H_2O_2 in 20 mM Tris/HCl (pH 7·5).

Preparation of polyclonal sera. *C. henricii* ATCC 15253^T, *B. vesicularis* LMG 2350^T and *C. maris* ATCC 15268^T were grown to OD_{600} of 0·3, washed three times in PBS, fixed in PBS with 2% formaldehyde, washed again three times in PBS, aliquoted and stored at −70 °C. A 1 ml aliquot corresponding to an OD_{600} of 0·3 was mixed with an equal volume of incomplete Freund's adjuvant (Sigma) and the emulsion was injected subcutaneously into a female rabbit. Four booster injections were given at intervals of 3 weeks and the animal was bled after 15 weeks (Harlow & Lane, 1988).

RESULTS

16S rRNA gene sequences

The 16S rDNA sequences of 62 strains described as species of the genus *Caulobacter* have been determined and analysed and three phylogenetic clusters were distinguished (Fig. 1). Three of the described species of *Caulobacter* (*C. crescentus*, *C. fusiformis* and *C. henricii*), all of them isolated originally from fresh-

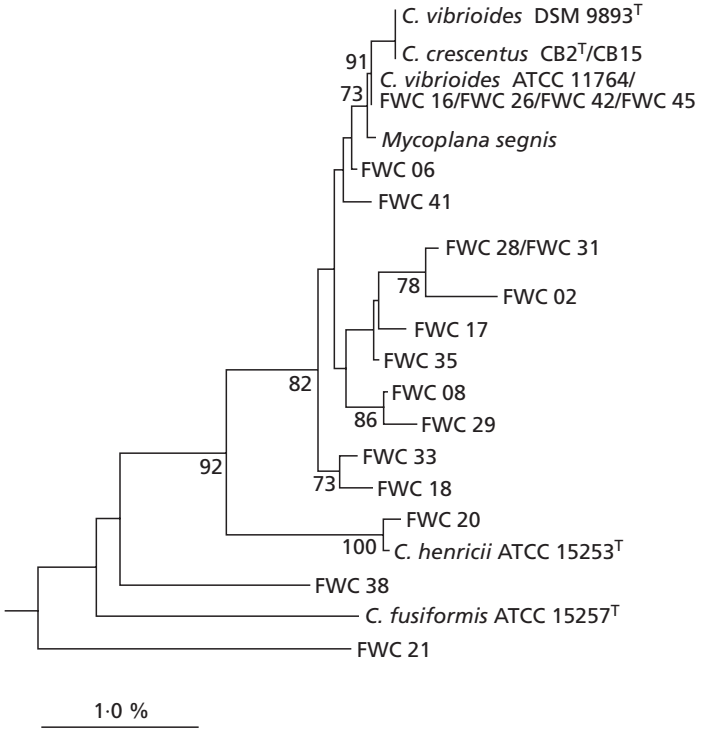


Fig. 2. Phylogenetic tree based on a comparison of 16S rDNA sequences of strains belonging to *Caulobacter sensu stricto*. The dendrogram was generated with the neighbour-joining algorithm of the PHYLIP package. The data set was resampled 100 times to obtain bootstrap values of confidence. Bootstrap proportions of confidence are shown for those branchings with values of more than 70 %.

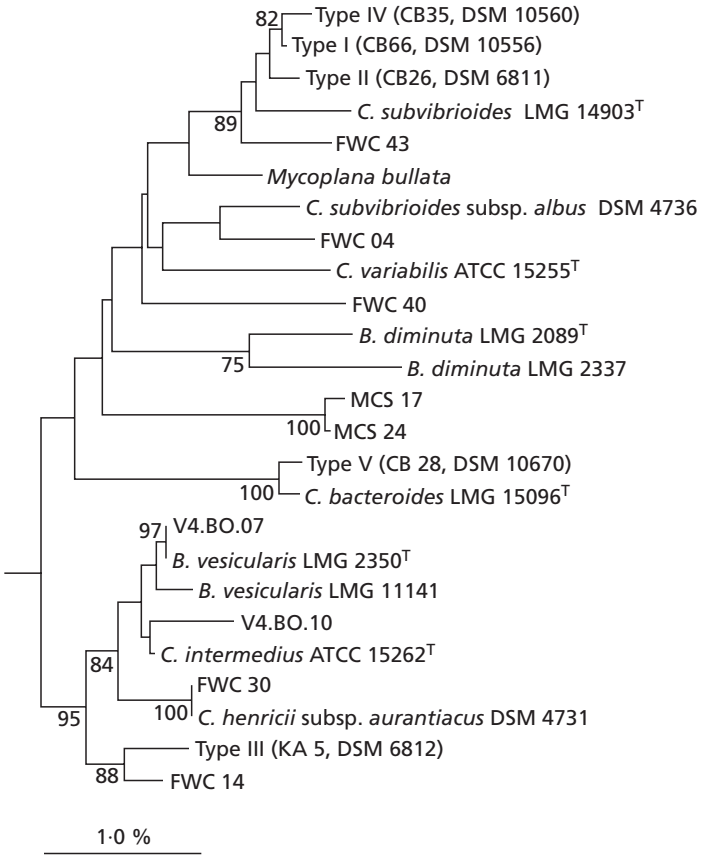


Fig. 3. Phylogenetic tree based on a comparison of the 16S rDNA sequences of strains belonging to *Brevundimonas.* For details see Fig. 2.

water, formed a monophyletic cluster with the type species of the genus, *C. vibrioides*. The 16S rDNA sequences of *C. vibrioides*, *C. crescentus* and a large number of freshwater isolates in this phylogenetic cluster, as well as *Mycoplana segnis*, were 99 % or more similar to each other, forming the '*C. vibrioides*-

Table 2. Similarity table (%) of 16S rDNA sequences of *Brevundimonas* (B) and *Caulobacter* (C) type strains

Strains (type species are given in bold): 1, ***Brevundimonas diminuta*** LMG 2089^T; 2, *Brevundimonas vesicularis* LMG 2350^T; 3, *Brevundimonas bacteroides* LMG 15096^T; 4, *Brevundimonas aurantiaca* comb. nov. DSM 4731^T; 5, *Brevundimonas intermedia* comb. nov. ATCC 15262^T; 6, *Brevundimonas subvibrioides* comb. nov. LMG 14903^T; 7, *Brevundimonas alba* comb. nov. ATCC 15265^T; 8, *Brevundimonas variabilis* comb. nov. ATCC 15255^T; 9, *Mycoplana bullata* DSM 7126^T; 10, ***Caulobacter vibrioides*** CB51^T (= VKM B-1496^T); 11, *Caulobacter crescentus* ATCC 15252^T; 12, *Caulobacter fusiformis* ATCC 15257^T; 13, *Caulobacter henricii* ATCC 15253^T; 14, ***Caulobacter vibrioides*** ATCC 11764; 15, *Caulobacter segnis* comb. nov. DSM 7131^T.

Genus	Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14
B	1	100·0													
B	2	97·0	100·0												
B	3	95·6	97·6	100·0											
B	4	96·7	99·2	97·4	100·0										
B	5	97·1	99·9	97·7	99·2	100·0									
B	6	96·8	97·3	96·2	96·8	97·4	100·0								
B	7	95·9	98·0	97·2	97·9	98·1	97·8	100.0							
B	8	95·9	97·2	97·9	96·8	97·2	98·0	98.1	100·0						
B	9	98·2	98·0	96·8	97·7	98·1	98·3	97.4	97·5	100·0					
C	10	93·7	94·9	93·5	94·9	95·0	93·5	93.6	93·4	93·7	100·0				
C	11	93·7									100·0	100·0			
C	12	94·3									96·5	96·5	100·0		
C	13	93·5									97·6	97·6	96·3	100·0	
C	14	93·9									99·8	99·8	96·7	97·8	100·0
C	15	93·8									99·8	99·8	96·7	97·9	99·9

assemblage' or '-lineage' (Fig. 2). Considering the 16S rDNA sequence data alone, these organisms, conceivably, could be assigned to a single species, although higher resolution methods, such as PCR typing, enable one to recognize distinct subclusters and to further resolve the assemblage. The 16S rDNA sequence data suggest that *C. crescentus* is synonymous with *C. vibrioides*, as the type strains of these species possessed identical 16S rRNA gene sequences.

C. henricii formed a distinct phylogenetic branch (Fig. 2), together with isolate FWC 20, which is yellow pigmented like *C. henricii* and probably represents another strain of *C. henricii*.

C. fusiformis comprised another distinct branch outside the '*C. vibrioides*-assemblage' (Fig. 2), as did the freshwater isolates FWC 21 and FWC 38, both representing as yet undescribed species. An important note is that the 16S rDNA sequence determined for *C. fusiformis* (ATCC 15257^T) in this study was identical to the sequence published previously (Stahl *et al.*, 1992) for strain 'CB7', i.e. the type strain of *C. bacteroides*. The 16S rDNA sequence determined in this study for *C. bacteroides* (LMG 15096^T) clustered with those of species of the genus *Brevundimonas*.

The 16S rDNA sequences of four described species classified as *Caulobacter* (*C. bacteroides*, *C. intermedius*, *C. subvibrioides* and *C. variabilis*) clustered with those of the described species of *Brevundimonas*. *B. diminuta* and *B. vesicularis* formed distinct lineages within the cluster (Fig. 3). The 16S rDNA sequence data demonstrated that *B. diminuta* strain LMG 2337 is distinct from the type strain of *B. diminuta* and probably represents a new species of *Brevundimonas*. *C. bacteroides*, *C. subvibrioides* and *C. variabilis* fall within the *Brevundimonas* phylogenetic cluster, with 16S rDNA sequence similarity values of 93·5, 93·5 and 93·4 %, respectively, to *C. vibrioides*, the type species of the genus *Caulobacter*. The 16S rDNA sequence of *C. intermedius* was 95 % similar to that of *C. vibrioides* but was 99·2 % similar to the 16S rDNA sequence of *B. vesicularis* (Table 2). Clearly, in light of these data, *C. bacteroides*, *C. intermedius*, *C. subvibrioides* and *C. variabilis* cannot be considered to be species of *Caulobacter*.

Isolates MCS 17 and MCS 24, with 99·8 % 16S rDNA sequence similarity, formed a distinct branch within the *Brevundimonas* cluster, representing a novel species of the genus. Further, on the basis of the 16S rDNA sequence data, strains FWC 04, FWC 40 and FWC 43 may all represent new species. *C. henricii* subsp. *aurantiacus* together with isolate FWC 30, which both have identical 16S rDNA sequences and are yellow pigmented, possess a high similarity to the 16S rDNA sequence of *B. vesicularis*, but form a distinct branch. They may represent another species of *Brevundimonas*. The 16S rDNA sequence data revealed that *C. subvibrioides* subsp. *albus* is phylogenetically too divergent to represent a subspecies of *C. subvibrioides*, as the dissimilarity of the 16S rDNA sequences of these strains was 2·2 %, supporting the DNA–DNA hybridization data (Moore *et al.*, 1978).

The organisms denoted as Types I–V have been

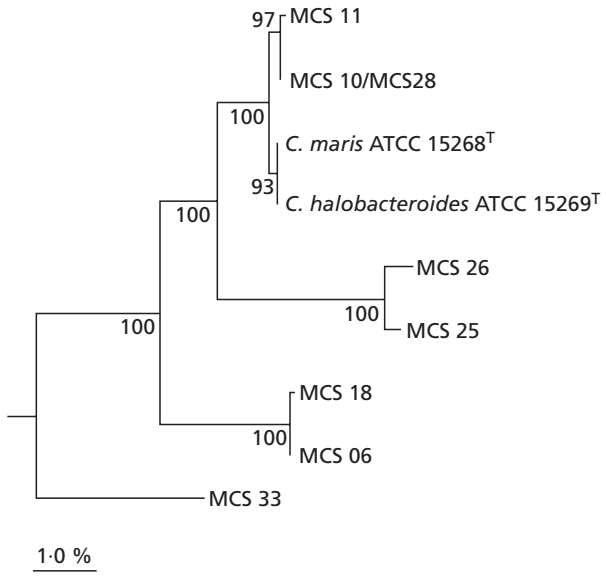


Fig. 4. Phylogenetic tree based on a comparison of the 16S rDNA sequences of strains belonging to *Maricaulis* gen. nov. For details see Fig. 2.

characterized by Poindexter (1964) and were distinct from the *Caulobacter* species described in that publication, although no species epithet was assigned to these organisms. The five representatives of the respective Types I–V deposited in public strain collections clustered phylogenetically with the species of *Brevundimonas*.

The marine species of *Caulobacter*, *C. maris* and *C. halobacteroides*, were observed to form a distinct phylogenetic cluster (i.e. *Maricaulis* gen. nov.), including seven of the MCS isolates (Fig. 4). Phylogenetically, these organisms were more related to species of the genus *Hyphomonas* than to species of *Caulobacter sensu stricto*. The 16S rDNA sequences of *C. maris* and *C. halobacteroides* were identical and very similar to the sequences of isolates MCS 10, MCS 11 and MCS 28. Marine isolates MCS 25 and MCS 26, on the one hand, and MCS 6 and MCS 18, on the other hand, formed pairs composed of closely related strains with high 16S rDNA sequence similarities, indicating that they may represent two additional species of *Maricaulis* gen. nov.

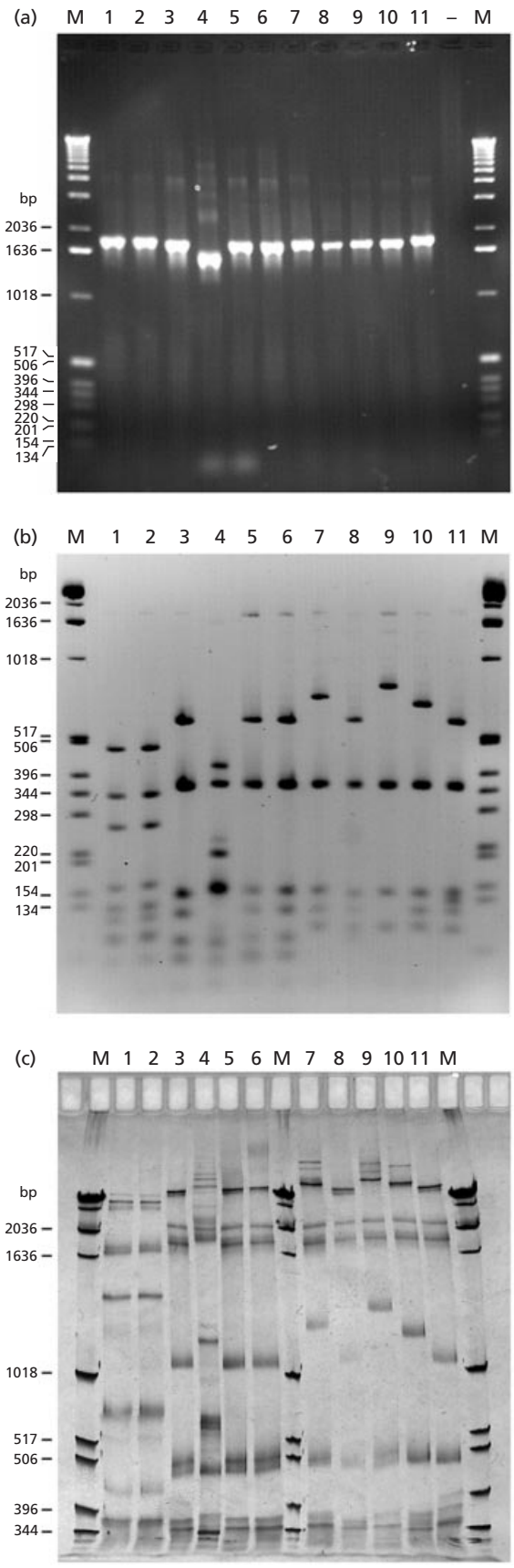
The 16S rDNA sequence of MCS 33 revealed a similarity of only 94·2 % to that of *C. maris*, possibly identifying it as a member of a separate, undescribed genus. The 16S rDNA sequence of strain MCS 23 had similarity values of 88·4 % to the sequence of *C. maris*, 86·1 % to the sequence of *C. vibrioides* and 90·4 % to the sequence of *Hyphomonas polymorpha*. These levels of 16S rDNA sequence similarity indicate that strain MCS 23 represents a distinct phylogenetic lineage separate from any described species. MCS 23 is more related to species of *Hyphomonas* than to *Maricaulis* gen. nov. and probably represents a member of yet another new genus.

The 16S rDNA sequence of *C. leidyia* did not group with any of the three phylogenetic clusters described above. While the similarity of the 16S rDNA sequence of *C. leidyia* to that of *C. vibrioides* was only 86·9 %, *C. leidyia* had 94·9 % 16S rDNA sequence similarity to *Sphingomonas paucimobilis*, the type species of the genus, and was most similar (96·7 %) to the 16S rDNA sequence of *Sphingomonas adhaesiva*. Therefore, on the basis of 16S rDNA sequence analysis of this study, *C. leidyia* may be recognized as a species of the genus *Sphingomonas*, which is also a genus within the α-subclass of the *Proteobacteria*, but phylogenetically distinct from *Caulobacter*. The 16S rDNA sequence for *C. leidyia* determined in this study was identical to that (GenBank accession no. M83797) described as being from *C. subvibrioides* strain CB81^T (Stahl *et al.*, 1992). However, from the data obtained in this study, the 16S rDNA sequence of *C. subvibrioides* (LMG 14903^T = ATCC 15264^T = CB81^T) was clearly different from the sequence reported by Stahl *et al.* (1992) and, in an independent study, Sly *et al.* (1997) also recognized that the 16S rDNA sequence for *C. subvibrioides* (ATCC 15264^T = ACM 2483^T = CB81^T) is different from that published by Stahl *et al.* (1992). The sequence determined in this study for *C. subvibrioides* was identical with that determined by Sly *et al.* (1997).

Analysis of 16S–23S ISR

The 16S–23S rDNA ISR has been used as a rapid typing tool for differentiating closely related *Caulobacter* species and isolates. For this purpose, primers complementary to highly conserved regions in the 3′ part of the 16S rRNA gene (the forward primer) and the 5′ part of the 23S rRNA gene (the reverse primer) were used for PCR amplification. The reference strains and isolates analysed possessed differences, as demonstrated by electrophoretic mobilities, primarily in the lengths of the PCR-amplified ISR (Fig. 5a). All strains of *Caulobacter* species displayed a PCR product of a single high-molecular-mass band ranging in size from 1·4 kb (*C. vibrioides* ATCC 11764) to 1·8 kb (*C. maris* ATCC 15266^T and *C. halobacteroides* ATCC 15269^T), with approximately 0·6 kb corresponding to the 3′ part of the 16S rDNA, nearly 0·5 kb corresponding to the 5′ part of the 23S rDNA, and the difference, 0·3–0·7 kb, comprising the 16S–23S rDNA ISR.

Differences in the sizes of PCR-amplified ISR products allowed the differentiation of the marine species, *C. maris* and *C. halobacteroides*, as one type, from the organisms comprising closely related (by 16S rDNA sequence comparisons) organisms clustering around the type strain of *C. vibrioides* (Fig. 2). Further, a higher-resolution approach, comprising the fingerprint analysis of fragments from restriction digestion of the ISR PCR products, supported the typing of the strains,



as defined by the size differentiation of the ISR PCR products. After *Taq*I digestion, ISR fingerprints of higher complexity were obtained, as expected (Fig. 5b). The ISR PCR products of the type strains of *C. maris* and *C. halobacteroides* produced exactly the same pattern after *Taq*I digestion and were easily distinguishable from the other strains. *C. vibrioides* DSM 9893^T (as well as VKM B-1496^T) and *C. crescentus* CB2^T also exhibited an identical ISR *Taq*I fingerprint pattern, while variations in the fingerprints of the other strains were observed, mainly at the highest molecular mass bands. These high-molecular-mass ISR restriction bands of *C. vibrioides* DSM 9893^T (and also VKM B-1496^T), *C. crescentus* CB2^T and isolates FWC 16 and FWC 45 possessed identical mobilities while *C. crescentus* CB15 and isolates FWC 26 and FWC 42 possessed high-molecular-mass ISR restriction bands, in each case, of unique electrophoretic mobility. A common ISR restriction band of 0·3–0·4 kb was observed in all strains of *C. vibrioides*, *C. crescentus* and FWC isolates analysed and was absent in the marine strains of *C. maris* and *C. halobacteroides*. Although *C. vibrioides* ATCC 11764 was observed to possess an overall ISR fingerprint pattern different from all other strains, it did share two bands common to all strains of *C. vibrioides*, *C. crescentus* and FWC isolates analysed.

Finally, SSCP analysis was applied to the *Taq*I restriction fragments. Under the conditions described in the Methods, no additional distinctions were detected between strains of the same types, as defined by the sizes of ISR PCR products and by *Taq*I restriction patterns of ISR PCR products, (Fig. 5c), indicating that no microheterogeneity exists in the nucleotide sequences of the respective fragments.

DNA–DNA hybridization

Because identical 16S rDNA sequences were determined for *C. maris* and *C. halobacteroides* and no differences in the ISR region of these species were found, DNA–DNA hybridizations between *C. maris* ATCC 15268^T and *C. halobacteroides* ATCC 15269^T were performed in triplicate. For both type strains, a DNA binding value of 92 % was found.

Fig. 5. (a) Fingerprints generated by ISR-PCR of *Caulobacter* strains and new isolates in 1·5 % agarose gels. (b) *Taq*I restriction analysis of PCR-amplified ISR of the different strains in 3·0 % agarose gels. (c) SSCP gel of the PCR-amplified ISR after digestion with *Taq*I. Samples in all the gels were loaded as follows: 1, *C. maris* ATCC 15268^T; 2, *C. halobacteroides* ATCC 15269^T; 3, *C. vibrioides* DSM 9893^T; 4, *C. vibrioides* ATCC 11764; 5, *C. crescentus* CB2^T; 6, *C. vibrioides* VKM B-1496^T; 7, *C. crescentus* CB15; 8, *Caulobacter* sp. FWC 16; 9, *Caulobacter* sp. FWC 26; 10, *Caulobacter* sp. FWC 42; 11, *Caulobacter* sp. FWC 45. In (a), a negative control for ISR PCR amplification was included (–). M, 1 kb DNA ladder molecular mass marker (Gibco-BRL).

Respiratory lipoquinones

Examination of all strains listed for their respiratory lipoquinone composition indicated that only ubiquinones were present. Separation of the ubiquinones according to the length of the isoprenoid side-chain indicated that all organisms contained ubiquinone (Q-10) as the major respiratory quinone.

Phenotypic characterization

Caulobacteria which cluster together by 16S rDNA sequence analysis and lipid patterns showed similar salt tolerances (Fig. 6). *Caulobacter crescentus*, *C. vibrioides* and *C. fusiformis* showed reduced growth without salt, optimal growth with 5 g NaCl l^{-1} and no growth at salt concentrations of 10 g l^{-1} or more (Fig. 6a). Contrary to these strains, the bacteria clustering around the type strains of *Brevundimonas* grew without salt, tolerated salt concentrations up to 20 g l^{-1} and did not grow above 80 g salt l^{-1}. The diversity observed in the 16S rDNA sequences and lipid data of the *Brevundimonas* cluster was mirrored in the tolerance to salt, resulting in a broad range of different tolerances of the individual species to salt concentrations between 20 and 80 g l^{-1} (Fig. 6b). Species of *Maricaulis* gen. nov., on the other hand, showed no or only reduced growth without salt, reached their optimal growth rates between 20 and 80 g l^{-1} and showed no or reduced growth at 100 g salt l^{-1} (Fig. 6c). This overall difference in salt tolerance was also observed with marine caulobacteria allowing identification of the isolates as *Brevundimonas* or *Maricaulis* gen. nov. Although *Caulobacter maris* and *C. halobacteroides* were differentiated in their description by their different tolerances to salt (Poindexter, 1964), we observed nearly identical salt tolerance.

Because the *Brevundimonas* strains and *Mycoplana bullata* and *Mycoplana segnis* were so different from *Caulobacter* spp. in terms of morphology but closely related in terms of molecular data, we looked for the presence of prosthecae. We grew *B. diminuta* LMG 2089^T and *Mycoplana bullata* DSM 7126^T in medium PYEM containing only 0·2 g peptone/yeast extract l^{-1}. Neither species developed prosthecae, whether grown in PYEM medium or in this modified PYEM medium.

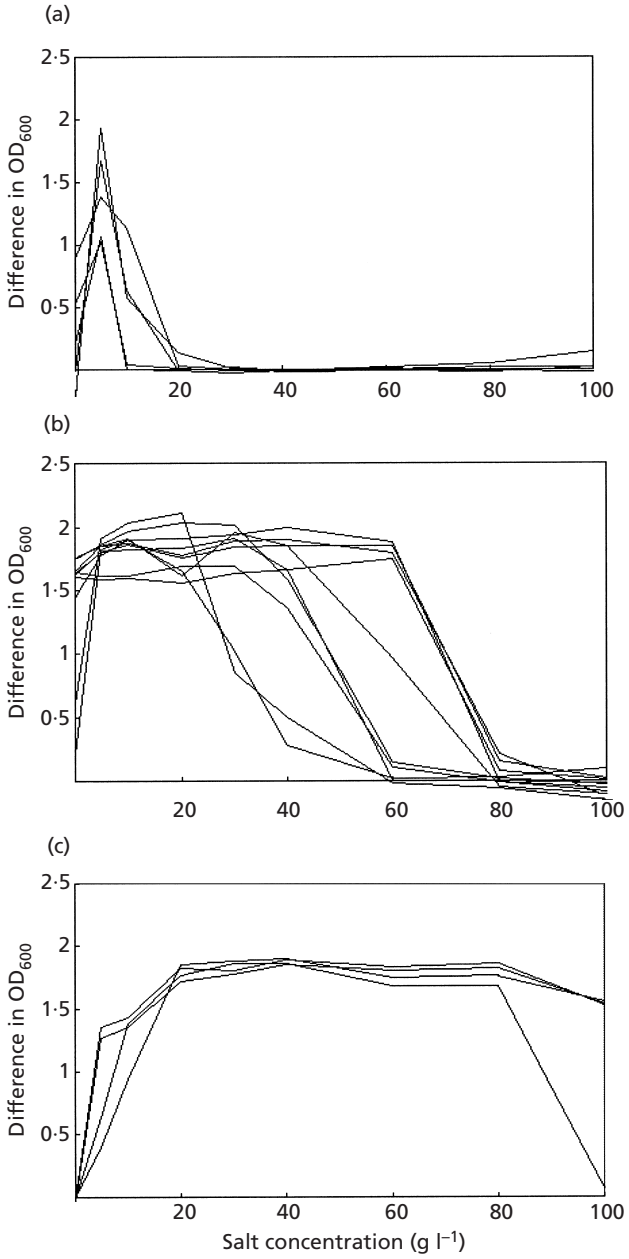


Fig. 6. Growth of strains of caulobacteria at different salt concentrations. (a) Growth of strains belonging to the genus *Caulobacter sensu stricto*. (b) Growth of type strains of *Brevundimonas* at different salt concentrations. Note the broad range of salt tolerances of the different species. (c) Growth of type strains and some isolates of *Maricaulis* gen. nov. at different salt concentrations.

Analysis of fatty acid methyl esters

The cellular fatty acid compositions of *Caulobacter* type strains and type strains of phylogenetically related taxa are shown in Table 3. The caulobacteria could be divided into four major groups. One, with *Caulobacter* subsp. *sensu stricto*, contained significant amounts of the fatty acids 12:1 3-OH, 14:0, 16:0, sum4 (Table 3), sum7 (Table 3) and ECL 18.080. All strains, except *Mycoplana segnis*, additionally contained ECL 11.798, 15:0, 17:0, 17:1ω6*c* and 17:1ω8*c*. Closely related to this group was the second group with *Brevundimonas* and related strains. Here, as in *Caulobacter sensu stricto*, 14:0 was present which was lacking in all other groups. The *Brevundimonas* group differed from all other groups by the presence of an unidentified fatty acid at ECL 17.897. Fatty acids present in all *Brevundimonas* strains were 12:0 3-OH, 14:0, 15:0, 16:0, sum4, 17:0, 17:1ω6*c*, 17:1ω8*c* and sum7. *Brevundimonas* strains are differentiated from *Caulobacter* strains by the absence of ECL 11.789 and by the presence of at least traces of ECL 17.897, only traces of 12:1 3-OH, higher amounts of 12:0 3-OH and by a

Table 3. Fatty acid content (mean percentage of total) of whole-cell hydrolysates of *Caulobacter* strains

Only the fatty acids counting for more than 1·0 % (mean amount) were indicated. The following strains contained significant amounts (> 1·0 %) of additional fatty acids: *B. diminuta* LMG 2089^T and LMG 2337 also contained 19:0cycloω8*c* (6·2 and 7·4 %, respectively); *C. leidyia* ATCC 15260^T also contained 16:0 2-OH (1·7 %) 18:1ω5*c* (2·4 %) and 19:0cycloω8*c* (1·5 %); *C. subvibrioides* LMG 14903^T also contained 20:2ω6,9*c* (1·6 %); *Caulobacter* sp. FWC 38 also contained 20:0 (1·4 %); *H. polymorpha* DSM 2665^T also contained sum9 (19:0cycloω10*c* and/or ECL 18.846 and/or ECL 18.858; 4·5 %); *Mycoplana bullata* DSM 7126^T also contained 19:0cycloω8*c* (1·1 %); *C. halobacteroides* ATCC 15269^T also contained 16:1ω5*c* (1·3 %); *C. maris* ATCC 15268^T also contained 16:1ω9*c* (1·0 %); *C. subvibrioides* subsp. *albus* also contained ECL 15.275 (2·0 %), 16:1ω9*c* (2·7 %) and 19:0cycloω8*c* (2·4 %); *Mycoplana segnis* LMG 17158^T also contained ECL 18.140 (6·9 %). ECL, unidentified fatty acid with equivalent chain-length. Sum, Summed feature. Sum4 consisted of one or more of the following fatty acids which could not be separated by the Microbial Identification System: 15:0iso 2-OH, 16:1ω7*c* and 16:1ω7*t*. Sum7 consisted of one or more of the following fatty acids: 18:1ω7*c*, 18:1ω9*t* and 18:1ω12*t*. Strains: 1, *C. bacteroides* LMG 15096^T; 2, *C. henricii* subsp. *aurantiacus* ATCC 15266^T; 3, *C. intermedius* ATCC 15262^T; 4, *C. subvibrioides* LMG 14903^T; 5, *C. subvibrioides* subsp. *albus* ATCC 15265^T; 6, *C. variabilis* ATCC 15255^T; 7, *B. vesicularis* LMG 2350^T; 8, *B. diminuta* LMG 2089^T; 9, *Mycoplana bullata* DSM 7126^T; 10, *C. crescentus* CB2^T; 11, *C. fusiformis* ATCC 15257^T; 12, *C. henricii* ATCC 15253^T; 13, *C. vibrioides* ATCC 11764; 14, *Mycoplana segnis* LMG 17158^T; 15, *C. maris* ATCC 15268^T; 16, *C. halobacteroides* ATCC 15269^T; 17, *Caulobacter* sp. FWC 38; 18, *C. leidyia* ATCC 15260^T.

Strain	Medium	11:0iso 3-OH	ECL 11.798	12:0	12:0 3-OH	12:1 3-OH	14:0	14:0 2-OH	15:0	16:0	Sum4	16:1 2-OH
Genus *Brevundimonas*												
1	PYEM	–	–	–	2·3	–	3·0	–	TR	12·8	6·7	–
2	PYEM	–	–	–	2·3	TR	3·4	–	3·0	21·3	4·0	–
3	PYEM	–	–	–	1·7	–	1·5	–	2·8	24·3	7·5	–
4	PYEM	–	–	–	2·8	–	4·6	–	1·5	15·9	11·2	–
5	PYEM	–	–	–	1·1	TR	1·0	–	5·4	16·8	5·9	–
6	PYEM	–	–	–	2·3	–	2·5	–	5·4	13·5	6·2	–
7	PYEM	–	–	–	1·9	–	2·4	–	4·0	20·7	5·1	–
8	PYEM	–	–	–	1·5	TR	TR	–	7·6	10·1	1·0	–
9	PYEM	–	–	–	1·3	TR	TR	–	5·1	18·9	4·5	–
Genus *Caulobacter sensu stricto*												
10	PYEM	–	1·4	–	TR	1·0	1·4	TR	13·6	13·3	8·6	6·1
11	PYEM	–	TR	1·9	–	2·8	1·3	–	13·8	11·7	TR	–
12	PYEM	–	1·8	–	TR	1·2	2·6	–	14·9	12·7	19·3	–
13	PYEM	–	2·4	TR	TR	1·6	1·5	–	7·1	15·4	14·9	–
14	PYEM	–	–	3·5	2·4	2·7	2·7	–	–	20·2	19·7	5·0
Genus *Maricaulis*												
15	SPYEM	2·6	–	–	–	–	–	–	TR	17·0	6·6	–
16	SPYEM	3·8	–	–	–	–	–	–	TR	15·6	6·0	–
Ungrouped strains												
17	PYEM	–	–	2·7	–	3·4	TR	–	3·9	16·5	TR	–
18	PYEM	–	–	–	–	–	–	13·3	–	16·6	–	–

Strain	17:0	17:1 ω6*c*	17:1 ω8*c*	17:0 iso	17:1 isoω9*c*	ECL 17.897	18:0	Sum7	18:1 ω9*c*	ECL 18.080	ECL 18.424	ECL 18.797
Genus *Brevundimonas*												
1	TR	TR	1·4	–	–	TR	TR	69·4	–	TR	–	–
2	1·7	1·0	1·6	–	–	1·3	TR	56·5	–	3·5	–	–
3	1.6	1.1	2.0	–	–	TR	TR	49.4	–	6.3	–	–
4	TR	TR	1·8	–	–	TR	1·4	56·7	–	TR	–	–
5	2·5	1·5	7·7	–	–	1·4	TR	43·2	1·0	2·7	–	TR
6	2·2	2·5	5·7	–	–	TR	TR	55·8	–	–	–	1·0
7	2·4	1·4	2·6	–	–	1·1	TR	53·7	–	4·5	–	–
8	8·5	6·2	10·8	–	–	1·1	TR	38·7	–	–	–	3·1
9	1·6	1·5	3·3	–	–	1·1	TR	55·5	–	1·6	–	TR
Genus *Caulbacter sensu stricto*												
10	6·3	4·0	4·6	–	–	–	TR	32·3	–	5·9	–	TR
11	7·1	8·9	4·0	–	–	–	–	42·4	–	4·8	–	–
12	1·6	3·2	5·3	–	–	–	TR	35·7	–	TR	–	–
13	1·9	1·6	2·6	TR	TR	–	TR	39·9	–	6·3	–	TR
14	–	–	–	–	–	–	–	35·4	–	1·5	–	–
Genus *Maricaulis*												
15	5·3	TR	4·0	7·7	17·4	–	1·1	24·5	6·4	1·6	1·3	TR
16	6·2	TR	4·8	7·1	16·9	–	1·1	24·0	6·2	1·6	1·3	TR
Ungrouped strains												
17	8·1	1·3	1·4	–	–	–	TR	18·6	–	40·5	–	TR
18	–	2·3	–	–	–	–	–	59.0	–	4·2	–	–

–, Not detected; TR, trace amount (< 1·0 %).

Table 4. Polar lipids as biomarkers in caulobacteria, *Hyphomonas* and *Sphingomonas* (found in strains listed in Table 1)

PC, Phosphatidylcholine; PG, phosphatidylglycerol; SQDG, 1,2-diacyl-3-*O*-sulfoquinovosylglycerol; TAU, 1,2-diacyl-3-α-D-glucuronopyranosyl-*sn*-glycerol taurine amide; DGL, 1,2-di-*O*-acyl-3-*O*-[D-glucopyranosyl-(1 → 4)-α-D-glucopyranuronosyl]glycerol; PGL, 1,2-diacyl-3-*O*-[6′-phosphatidyl-α-D-glucopyranosyl]glycerol; MGD, 1,2-di-*O*-acyl-3-*O*-α-D-glucopyranosyl glycerol; MGDOx, 1,2-di-*O*-acyl-3-*O*-α-D-glucopyranuronosyl glycerol. + + +, Present in all strains; + +, present in most strains (> 80%); blank, absent.

Genus/strain	PC	PG	SQDG	TAU	DGL	PGL	MGD	MGDOx
Caulobacter		+ + +				+ + +	+ + +	+ + +
Brevundimonas		+ + +	+ +		+ +	+ + +	+ + +	+ + +
Maricaulis		+ + +	+ + +	+ +			+ + +	+ + +
Hyphomonas		+ + +		+ + +			+ + +	+ + +
MCS 23		+ + +		+ + +			+ + +	+ + +
MCS 33							+ + +	+ + +
Sphingomonas	+ + +	+ + +						

higher content of the dominant fatty acid sum7. The *Maricaulis* gen. nov. group was the only one where 11:0iso 3-OH, 17:0iso, 17:1isoω9*c* and 18:1ω9*c* were present and 12:0 3-OH and 12:1 3-OH were absent. Contrary to the other groups, 15:0 was found only in traces. The fourth group comprised *Hyphomonas* strains with considerable amounts of 17:0 and an unidentified fatty acid at ECL 18.797. *C. leidyia* differed from all these strains by the presence of large amount of 14:0 2-OH. *Caulobacter* sp. FWC 38 could also not be grouped mainly because of the huge amounts of *trans*-11-methyl-12-octadecenoic acid (ECL 18.080) found whereas sum7 (see Table 3) was considerably reduced.

Mass spectrometric analysis of polar lipids (glyco- and phospholipids)

The polar lipids were analysed by chromatographic and spectroscopic methods. ^{1}H-NMR spectra of the polar lipid fractions of almost all strains revealed the presence of poly-β-hydroxybutyric acid. Individual polar lipids were analysed and their structures elucidated using tandem mass spectrometry (Abraham *et al.*, 1997). From the strains included in this study, 26 different phosphoglucolipids could be identified. Although more than 30 different fatty acids were detected in the phospholipid fractions of *Caulobacter*, *Brevundimonas* and *Mycoplana* spp., only seven different fatty acids occurred within these 26 different phosphoglucolipids. Furthermore, three different sulfoquinovosyl diacylglycerols were detected. This lipid type occurred in all halophilic *Caulobacter* strains, in most *Brevundimonas* strains but only in few freshwater *Caulobacter* strains, while all *Hyphomonas* strains lacked these sulfolipids (Batrakov *et al.*, 1996; Abraham *et al.*, 1997). Glucuronotaurine amides were found only in the halophilic *Caulobacter* and *Hyphomonas* strains but were absent from the freshwater strains of *Caulobacter* and *Brevundimonas* and the halophilic bacterium MCS 33. The distribution of the different types of phospholipids, especially the sulfoquinovosyl diacylglycerols, glucuronotaurine amide diacylglycerols and phosphatidyl glucopyranosyl diacylglycerols, led to the identification of four different phenotypic groups of these bacteria, in agreement with the phylogenetic groupings published previously (Stahl *et al.*, 1992) and the groupings derived from 16S rRNA gene sequences in this study. One group comprised *Caulobacter sensu stricto* with *C. vibrioides*. The second group comprised the type strains of *Brevundimonas*, including the type species *B. diminuta* and a considerable number of described *Caulobacter* spp. The third group included the halophilic *Caulobacter* spp. around *C. maris*. The latter group lacked the phosphatidyl glucopyranosyl diacylglycerols (present both in *Caulobacter sensu stricto* and *Brevundimonas* strains) but contained sulfoquinovosyl and glucuronotaurine amide diacylglycerols. They could be discerned from the fourth group, *Hyphomonas*, by the occurrence of sulfoquinovosyl diacylglycerols which were absent in strains of the genus *Hyphomonas*.

One characteristic of the genera analysed in this study was the high percentage of glycolipids within the polar lipids. Most strains contained higher amounts of glycothan phospholipids and some of them completely lacked phospholipids (Minnikin *et al.*, 1973). The glycolipids were α-D-glucopyranosyl diacylglycerol, α-D-glucopyranuronosyl diacylglycerol and α-D-glucopyranosyl-(1 → 4)-α-D-glucopyranuronosyl diacylglycerol (Wilkinson, 1969; Batrakov *et al.*, 1997). The mass spectrometric analysis of the glycolipids of 70 strains of *Caulobacter*, *Mycoplana*, *Hyphomonas* and *Brevundimonas* spp. has led to the identification of 60 different derivatives. A pattern of distribution of these glycolipids within the caulobacteria could be observed, where the glycolipids with a mass of 742 were more abundant in the halophilic caulobacteria than in any other group, *Hyphomonas* spp. contained more glucuronosyl lipids than the other groups and the occurrence of glucosyl glucuronosyl lipids was restricted to *Brevundimonas* spp. and closely

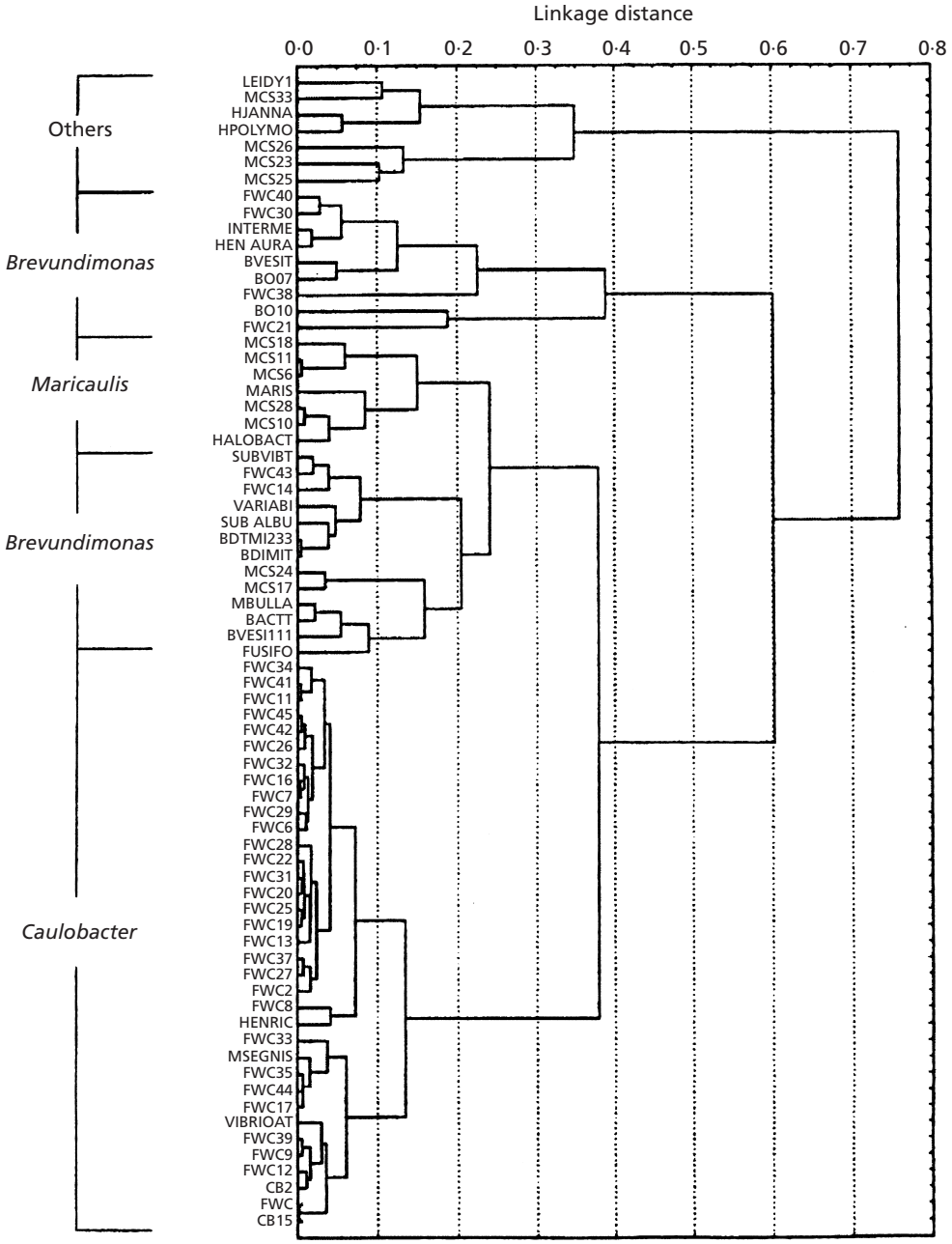


Fig. 7. Dendrogram derived from the relative intensities of the $[M+NH_4]^+$ ions between *m/z* 740–840 (most abundant ion set to 100 %). Unweighted pair group means and Pearson's coefficient were used to construct the dendrogram.

related strains (Table 4). DCI mass spectrometry using ammonia as ionization gas showed mainly the $[M+NH_4]^+$ ions and was used to compare the strains. The relative intensities of the $[M+NH_4]^+$ ions between *m/z* 740–840 were determined and compared by multivariate analysis. The dendrogram derived from these data revealed four groups of bacteria comprising *Caulobacter sensu stricto*, *Brevundimonas*, *Maricaulis* gen. nov. and a cluster containing *Hyphomonas* and other marine bacteria (Fig. 7).

Westprinting analysis of *Caulobacter* and *Brevundimonas* strains

To support the results of the 16S rDNA sequence and lipid analyses, an immunological approach has been taken for the identification of genus-specific antigens (Tesar *et al.*, 1996) among the *Caulobacter sensu stricto*, *Brevundimonas* and *Maricaulis* gen. nov. strains. For that purpose, a representative strain of each group was used to elicit antibodies in rabbits

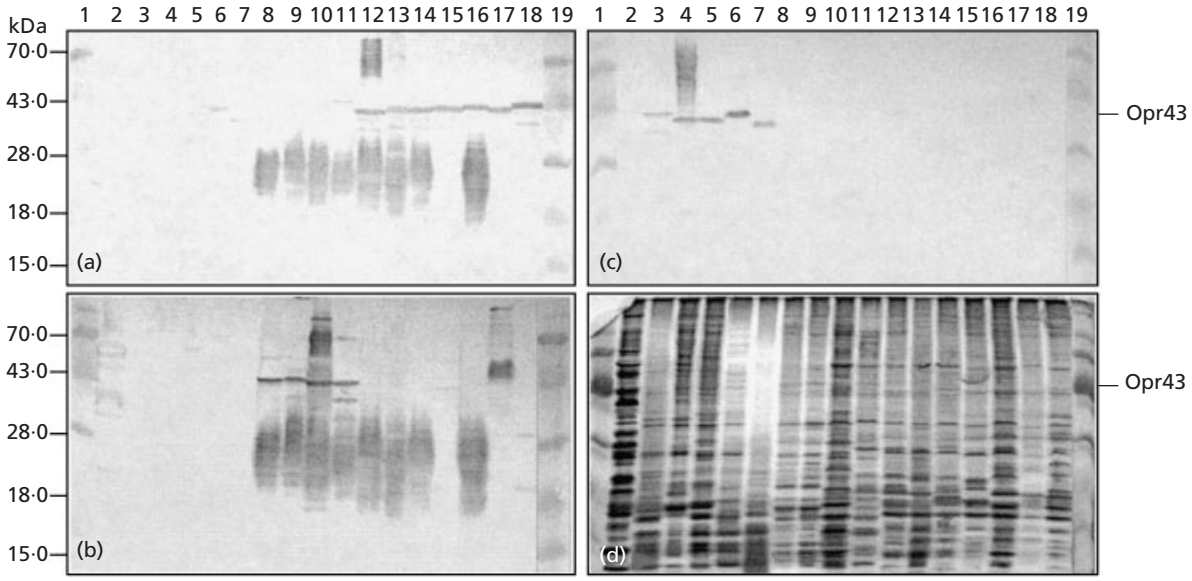


Fig. 8. Immunochemical analysis of different *Caulobacter* and *Brevundimonas* strains. Whole-cell lysates were subjected to SDS-PAGE followed by silver staining (d) or transfer to nitrocellulose membranes (a, b, c). Immunoblots were incubated with antisera SMT7 (a), SMT7 (b) and SMT25 (c). Bound antibodies were detected by a peroxidase-coupled anti-rabbit antiserum. Bacterial strains and collection reference numbers for each lane are as follows: 2, *C. leidyia* ATCC 15260^T; 3, *Caulobacter* sp. MCS 28; 4, *C. halobacteroides* ATCC 15269^T; 5, *Caulobacter* sp. MCS 25; 6, *Caulobacter* sp. MCS 26; 7, *Caulobacter* sp. MCS 18; 8, *C. vibrioides* ATCC 11764; 9, *C. crescentus* CB2^T; 10, *C. henricii* ATCC 15253^T; 11, *C. fusiformis* ATCC 15257^T; 12, *B. vesicularis* LMG 2350^T; 13, *C. intermedius* ATCC 15262^T; 14, *C. subvibrioides* LMG 14903^T; 15, *Mycoplana bullata* DSM 7126^T; 16, *C. alba* DSM 4736^T; 17, *C. bacteroides* LMG 15096^T; 18, *B. diminuta* LMG 2089^T; 1 and 19: Molecular masses (in kDa) of standards.

which could be used for group-specific immunological fingerprints in a Western blot. Three different antisera raised against *C. henricii* (serum SMT7), *B. vesicularis* (serum SMT2) and *Caulobacter maris* (serum SMT25) reacted exclusively with a subset of *Caulobacter* and *Brevundimonas* strains (Table 1). Using the reactivity against a band of 43 kDa in size as a group-specific biomarker (Fig. 8), the bacteria were clustered into three major groups. The first group of immunologically related strains, which reacted only with the serum SMT7, comprised freshwater isolates including the type strains of *C. vibrioides*, *C. crescentus*, *C. henricii* and *C. fusiformis* (Table 1). The second group of bacteria, which reacted exclusively with serum SMT2, showed a broader spectrum of strains isolated from different environments, including type strains *B. vesicularis* and *B. diminuta* as well as *C. intermedius*, *C. subvibrioides*, *C. bacteroides*, *Mycoplana bullata* and several freshwater and marine isolates (Table 1). A third group of *Caulobacter* strains which reacted exclusively with serum SMT25 included the type strains *C. maris*, *C. halobacteroides* and several other marine isolates (Table 1). These three groups fit with the genera *Caulobacter sensu stricto*, *Brevundimonas* and *Maricaulis* gen. nov. However, some strains described as *Caulobacter* isolates (MacRae & Smit, 1991; Walker *et al*., 1992) showed only a weak reaction with the antisera, as in the case of *Caulobacter* sp. FWC 5, FWC 21 and FWC 38, or no reaction, as in the case of *C. leidyia*. Further analysis of the 43 kDa biomarker showed that it was highly enriched in outer-membrane protein fractions (data not shown).

DISCUSSION

Sequence comparisons of the PCR-amplified 16S rRNA genes, lipid analyses and salt tolerances enabled us to group *Caulobacter* spp. *sensu lato* and some related species into three different clusters corresponding to taxonomic genus levels. One group comprised *Caulobacter sensu stricto*, including the type species *C. vibrioides* (i.e. the described type species of the genus), *C. henricii*, *C. fusiformis* and *Mycoplana segnis*. This genus was characterized by the absence of diglycosyl and sulfoquinovosyl lipids, the presence of phosphatidyl glucosyl lipids with the main mass at 1411 Da and minimal salt tolerances. The second genus comprised *Brevundimonas* spp., including the type species of *B. diminuta* and *B. vesicularis* as well as *C. bacteroides*, *C. henricii* subsp. *aurantiacus*, *C. intermedius*, *C. subvibrioides*, *C. subvibrioides* subsp. *albus*, *C. variabilis* and *Mycoplana bullata*. This group was characterized by the presence of diglycosyl, sulfoquinovosyl and phosphatidyl glucosyl lipids with main masses of 1413 and 1439 Da. These species tolerate salt at concentrations between 0–30 g NaCl l^{-1} (some species can grow in up to 60 g NaCl l^{-1}). A third group comprised the genus *Maricaulis* gen. nov. and included

C. maris and *C. halobacteroides*. This newly recognized genus was characterized by the presence of sulfoquinovosyl and glucuronopyranosyl taurine amide lipids, the absence of phosphoglucosyl lipid and salt tolerances between 10–60 g NaCl l^{-1} (some species can grow in up to 80 g NaCl l^{-1}). The occurrence of sulfoquinovosyl lipids in *Maricaulis* gen. nov. differentiated it from *Hyphomonas*, the species of which did not contain these lipids.

The genus *Mycoplana* is also paraphyletic, as shown by Yanagi & Yamasato (1993). According to their analysis of the 16S rDNA sequence of *Mycoplana dimorpha* IAM 13154^T, the type strain of the type species belonged to the family *Rhizobiaceae* while *Mycoplana bullata* grouped near *B. diminuta*. We propose to remove *Mycoplana segnis* and *Mycoplana bullata* from the genus *Mycoplana*, which is classified within the *Rhizobiaceae*, and to place these two species into the genera *Caulobacter* and *Brevundimonas*, respectively. Further investigations are needed to clarify the taxonomic position of *Mycoplana bullata* within the genus *Brevundimonas*.

C. crescentus could not be discerned clearly from *C. vibrioides* by the various methods described above. The genetic criterion, accepted internationally, for discerning different species is a DNA–DNA homology below 70%. Twenty years ago, Moore *et al.* (1978) reported the DNA–DNA homology between the neotype strain *C. vibrioides* CB51^T and *C. crescentus* CB2^T to be 97%, but they did not formally propose *C. crescentus* to be synonymous with *C. vibrioides*. Our results, especially the analysis of the 16S–23S ISR rDNA, further revealed that strains FWC 16 and FWC 45 were indistinguishable from *C. vibrioides* DSM 9893^T and *C. crescentus* CB2^T, whereas both *C. vibrioides* ATCC 11764 and *Caulobacter* sp. FWC 26 showed differences in their interspacer regions. Moore *et al.* (1978) performed DNA–DNA hybridizations between all described *Caulobacter* species including three subspecies. Apart from *C. vibrioides*, *C. crescentus* and *C. vibrioides* subsp. *limonus*, they found, for all type strains, DNA–DNA homologies well below 70%, identifying all of them as distinct species. Our results also proved that *C. maris* is conspecific with *C. halobacteroides* and a DNA–DNA homology of 92% further confirmed these results.

The analysis of the 16S rRNA genes of *C. vibrioides* DSM 9893^T and VKM B-1496^T showed that both sequences were identical. On the basis of this work, it could be concluded that strains of CB51^T, held in the DSMZ and in the VKM and obtained from two independent sources, were identical at the level of 16S rDNA. Thus, it has been possible to show that the type strain of *Caulobacter vibrioides*, which the '*Approved Lists of Bacterial Names*' (Skerman *et al.*, 1989) lists as being 'not extant', and which was the type species of the genus *Caulobacter*, is in fact held in the DSMZ and VKM. It is now possible to properly define the genera *Caulobacter* and *Brevundimonas*, for which *C. vibrioides* and *B. diminuta*, respectively, are the type species.

All methods of this study demonstrated that *C. leidyia* could not be a member of any of the taxa described above, but rather belongs to the genus *Sphingomonas*, although further studies concerning the genus *Sphingomonas* are required before a recombination of *C. leidyia* as a species of *Sphingomonas* can be made. We found the 16S rDNA sequences of *C. leidyia* strains ATCC 15260^T and DSM 4733^T to be identical to that of a strain wrongly labelled as *Caulobacter subvibrioides* (GenBank accession no. M83797; Stahl *et al.*, 1992). We also investigated the 16S rDNA sequence of the type strain of *C. subvibrioides* and found it to fit into the *Brevundimonas* cluster, as has also been reported by Sly (1997). Another confusion concerns the type strain of *C. bacteroides*, in that the 16S rDNA sequence determined by Stahl *et al.* (1992) was identical to the 16S rDNA sequence we have determined for *C. fusiformis* ATCC 15257^T, while the type strain of *C. bacteroides*, LMG 15096^T, appears in fact to be a member of the genus *Brevundimonas*.

The diverging polar lipids of some *Caulobacter* strains need some further discussion. *Caulobacter* sp. FWC 38 and *C. fusiformis* displayed lipids which were not found in other strains of the genus *Caulobacter sensu stricto*. The pattern of phosphoglycolipids of *Caulobacter* sp. FWC 38 was different from that of all other strains of both *Caulobacter* and *Brevundimonas*. The phosphoglycolipid with 1410 or 1412 Da is missing, but the phosphoglycolipid of 1438 Da, which is characteristic of the phospholipids determined for species of the genus *Brevundimonas*, was found. *C. fusiformis* had the phosphoglycolipid of 1412 Da but was otherwise similar to FWC 38. Both of them, however, did not contain diglycosyl lipids, another characteristic biomarker of the genus *Brevundimonas*. These results characterized these two strains at the margin of the genus *Caulobacter* near *Brevundimonas*. Surprisingly, *Caulobacter* sp. FWC 21 which is, according to the 16S rDNA data, even more remote from the 'core region' of the genus *Caulobacter* possessed a lipid pattern completely in agreement with the majority of *Caulobacter* strains.

Caulobacter strains have been compared based on the reactivity of an antiserum raised against the S-layer protein of *C. crescentus* CB15 (*rsaA* gene) (Walker *et al.*, 1992). However, the use of S-layer antisera is not especially suitable as a taxonomic aid since S-layers might be lost when grown under laboratory conditions (Koval *et al.*, 1991), and thus escape identification by immunological probes against S-layer proteins. Moreover, some freshwater strains and all marine strains lack an S-layer (Walker *et al.*, 1992; Anast & Smit 1988). Recently, a new strategy, termed 'Westprinting' (Western blot and immunological fingerprinting) has been described (Tesar *et al.*, 1996) which allows a systematic screening of genus- and strain-specific protein epitopes based on similar or identical protein

patterns among different strains of a taxon of interest. In this study, we used the Westprinting technique to identify antigenic epitopes among immuno-reactive proteins, which could serve as group-specific biomarkers for the identification of caulobacteria. Based on the reactivity of several antisera, a 43 kDa protein was identified as a protein biomarker which exhibited group-specific epitopes useful for the clustering of strains of *Caulobacter* and *Brevundimonas* into three different sub-groups, which was in good agreement with the 16S rDNA sequence and polar lipid analyses. Further studies revealed that the 43 kDa protein was highly enriched in the outer-membrane protein fraction. This contributes to the findings of others, that outer-membrane proteins share common immunogenic epitopes within a phylogenetically related group of bacteria (Hancock *et al.*, 1990; Ullstrom *et al.*, 1991; Kragelund *et al.*, 1996). However, the identity of the 43 kDa protein remains to be solved and further studies are in progress to analyse the N-terminal sequence for comparison with other outer-membrane proteins.

The results of this study demonstrated that cell morphology did not necessarily correlate with the phylogenetic relationships of bacteria. However, this general observation has been reported previously (e.g. Vandamme *et al.*, 1996). Our findings have revealed the important observation, that the morphologically characteristic 'prostheca' is paraphyletic. We could not see any prostheca in *B. diminuta* LMG 2089^T and *Mycoplana bullata* DSM 7126^T cells grown under conditions where all *Caulobacter* strains developed them. To date, only the newly proposed genus *Maricaulis* comprised entirely strains with prosthecae while *Brevundimonas* and *Caulobacter sensu stricto* contain both types. The presence of the prostheca-less species *Mycoplana segnis* in the genus *Caulobacter*, the membership of the prosthecate *C. bacteroides* or *C. intermedius* in the hitherto prostheca-less genus *Brevundimonas* or the prosthecate *C. leidyia* in the genus *Sphingomonas* demonstrate that either this morphology has developed several times during the evolution of the α-*Proteobacteria* and/or it was lost repeatedly. The observation that an isolate identified as *B. vesicularis* hybridized with a flagellin gene probe (Stahl *et al.*, 1992) suggests, at least in the case of the closely related *Brevundimonas* species, the possibility that these strains are locked in the motile phase.

Conclusions

Due to the heterogeneity of the genera *Caulobacter* and *Mycoplana*, certain recombinations are required and the proposal of the new genus *Maricaulis* for halophilic *Caulobacter* strains is necessary.

Caulobacter Henrici and Johnson 1935 emend.

The description of the genus *Caulobacter* given by Henrici & Johnson (1935) and emended by Poindexter (1964) must be further emended.

Gram-negative cells, rod-shaped, fusiform or vibroid, 0·4–0·5 by 1–2 μm. Cells usually possess a prostheca, but some species do not. They tolerate only small amounts of NaCl, grow without it, but some grow optimally with 5 g NaCl l^{-1}. No growth with salt concentrations above 20 g l^{-1} was observed. All strains contain significant amounts of the fatty acids 12:1 3-OH, 14:0, 16:0, sum4 (see Table 3), sum7 (see Table 3) and 11-Me 18:1ω5 (ECL 18.080). All strains, except *Mycoplana segnis*, additionally contain ECL 11.798, 15:0, 17:0, 17:1ω6*c* and 17:1ω8*c*. Polar lipids are α-D-glucopyranosyl diacylglycerol, α-D-glucopyranuronosyl diacylglycerol, 6-phosphatidyl-α-D-glucopyranosyl diacylglycerol (main mass number 1411 Da) and phosphatidylglycerol. The G+C content is 62–67 mol%. Type species is *Caulobacter vibrioides*.

Caulobacter vibrioides **Henrici and Johnson 1935 (syn. *Caulobacter crescentus* Poindexter 1964) (Moore *et al.*, 1978).** Neotype strain is CB51^T (= VKM B-1496^T). Conspecific are strains CB2^T, CB15 and CB16 (Moore *et al.*, 1978).

Caulobacter segnis **comb. nov.** Basonym: *Mycoplana segnis* Urakami *et al.* 1990. *Caulobacter segnis* is closely related to *C. vibrioides* according to 16S rRNA gene sequence similarity, but is clearly distinct from this species by morphology and cell cycle (Urakami *et al.*, 1990). Differs from *C. vibrioides* by the presence of ECL 18.140 and the lack of ECL 11.798, 15:0, 17:0, 17:1ω6*c* and 17:1ω8*c* in the whole-cell hydrolysate. Type strain is DSM 7131^T (= LMG 17158^T).

Brevundimonas Segers *et al.* 1994 emend.

Gram-negative cells, rod-shaped, fusiform, bacteroid or vibroid, 0·4–0·5 by 1–2 μm. Cells do not usually possess a prostheca, but some species do. They grow without NaCl, grow optimally with 5–20 g NaCl l^{-1}, growth is reduced at 30–80 g NaCl l^{-1} depending on the species, and they do not grow with NaCl concentrations above 80 g l^{-1}. Dominant fatty acids for all *Brevundimonas* strains are 12:0 3-OH, 14:0, 15:0, 16:0, sum4, 17:0, 17:1ω6*c*, 17:1ω8*c* and sum7. They are differentiated from *Caulobacter* strains by the absence of ECL 11.789 and significant amounts of 12:1 3-OH, by the presence of at least traces of ECL 17.897 and higher amounts of 12:0 3-OH, and by a higher content of the dominant fatty acid sum7 (see Table 3). Polar lipids are α-D-glucopyranosyl diacylglycerol, α-D-glucopyranuronosyl diacylglycerol, 1,2-di-*O*-acyl-3-*O*-[D-glucopyranosyl-(1→4)-α-D-glucopyranuronosyl]glycerol, 6-phosphatidyl-α-D-glucopyranosyl diacylglycerol (main mass numbers 1413 and 1439 Da), and phosphatidylglycerol. Most strains contain sulfoquinovosyl diacylglycerol. The G+C content is 65–68 mol%. Type species is *Brevundimonas diminuta*; type strain is LMG 2089^T.

Brevundimonas alba **comb. nov., stat. nov.** Basonym: *Caulobacter subvibrioides* subsp. *albus* Poindexter 1964. Closest relative is *Brevundimonas intermedia* to

which it is 98·1 % related by 16S rDNA similarity and 15 % by DNA–DNA homology (Moore *et al.*, 1978). Type strain is DSM 4736^T (= CB88^T).

***Brevundimonas aurantiaca* comb. nov., stat. nov.** Basonym: *Caulobacter henricii* subsp. *aurantiacus* Poindexter 1964. Closest relative is *B. intermedia* to which it is 99·2 % related by 16S rDNA similarity and 33 % by DNA–DNA homology (Moore *et al.*, 1978). Type strain is DSM 4731^T (= CB-R^T).

***Brevundimonas bacteroides* comb. nov.** Basonym: *Caulobacter bacteroides* Poindexter 1964. Closest relative is *B. variabilis* to which it is 97·9 % related by 16S rDNA similarity and 13 % by DNA–DNA homology (Moore *et al.*, 1978). Type strain is ATCC 15254^T (= CB7^T = LMG 15096^T).

***Brevundimonas intermedia* comb. nov.** Basonym: *Caulobacter intermedius* Poindexter 1964. Closest relative is *B. vesicularis* to which it is 99·9 % related by 16S rDNA similarity, but differs by morphology and cell cycle. Another close relative is *B. aurantiaca* to which it is 99·2 % related by 16S rDNA similarity and 33 % by DNA–DNA homology (Moore *et al.*, 1978). Type strain is ATCC 15262^T (= CB63^T).

***Brevundimonas subvibrioides* comb. nov.** Basonym: *Caulobacter subvibrioides* Poindexter 1964. Closest relative is *Mycoplana bullata* to which it is 98·3 % related by 16S rDNA similarity, but is clearly distinct from this species by morphology and cell cycle (Urakami *et al.*, 1990). Type strain is ATCC 15264^T (= CB81^T = LMG 14903^T).

***Brevundimonas variabilis* comb. nov.** Basonym: *Caulobacter variabilis* Poindexter 1989. Closest relative is *B. alba* to which it is 98·1 % related by 16S rDNA similarity and 1 % by DNA–DNA homology (Moore *et al.*, 1978). Type strain is ATCC 15255^T (= CB17^T).

Description of *Maricaulis* gen. nov.

Maricaulis (Ma.ri.cau′lis. L. neut. n. *mare* the sea; L. masc. n. *caulis* stalk referring to a prostheca; M.L. masc. n. *Maricaulis* stalk from the sea).

Gram-negative cells, rod-shaped, fusiform or vibroid, 0·4–0·5 by 1–2 μm. Cells possess a prostheca, ca. 0·15 μm in diameter and of varying length depending on the species and environmental conditions, extending from one pole as a continuation of the long axis of the cell. Adhesive material is present at the distal end of the prostheca. Occur singly. Multiplication by binary fission. At the time of separation one cell possesses a prostheca and the other a single polar flagellum. Each new appendage 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 prostheca at this site and enters the immotile vegetative phase. Colonies circular, convex, colourless. Chemo-organotrophic aerobes, but most of the strains tested could grow anaerobically probably using amino acid as fermentable carbon source (Anast & Smit, 1988). Nitrate is reduced to nitrite anaerobically by some strains. Most strains can store carbon as poly-β-hydroxybutyric acid. As well as mixtures of B vitamins and amino acids, other organic factors are required for growth. All strains can grow on peptone yeast extract media with 5 g NaCl l^{-1}; optimal growth between 20–60 g NaCl l^{-1}. NaCl is required for optimal growth, only a few strains tolerate salt concentrations above 100 g NaCl l^{-1}. Growth is inhibited or cells become deformed in media containing 1 % (w/v) or more organic material. Temperature range of most isolates is 15–35 °C; 20–25 °C is optimal. The optimal pH for growth is around neutrality, pH range is 6·0–8·0. All strains are characterized by three major fatty acids 16:0, 17:1isoω9c and sum7 (see Table 3) and the following minor compounds: 11:0iso 3-OH, 17:1ω8, 17:0iso, 17:0, and 18:1ω9. Polar lipids are α-D-glucopyranosyl diacylglycerol, α-D-glucopyranuronosyl diacylglycerol, sulfoquinovosyl diacylglycerol, α-D-glucuronopyranosyl diacylglycerol taurine amide. Most strains contain also phosphatidylglycerol. All isolates were obtained only from sea water. The G+C content is 62·5–64·0 mol %. The type species is *Maricaulis maris*.

***Maricaulis maris* comb. nov.** Basonym: *Caulobacter maris* Poindexter 1964. Type strain is ATCC 15268^T (= CM 11^T). The G+C content is 62·5 mol %. Synonym: *Caulobacter halobacteroides* Poindexter 1964 ATCC 15269^T (= CM 13^T) (DNA–DNA homology between the two type strains is 92 %).

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