Phylogeny by a polyphasic approach of the order *Caulobacterales*, proposal of *Caulobacter mirabilis* sp. nov., *Phenylobacterium haematophilum* sp. nov. and *Phenylobacterium conjunctum* sp. nov., and emendation of the genus *Phenylobacterium*

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Three strains of Gram-negative, rod-shaped, non-spore-forming bacteria were isolated from fresh water and human blood. As determined by analyses of 16S rRNA gene sequences, the prosthecate strain FWC 38^T was affiliated to the alphaproteobacterial genus *Caulobacter*, with *Caulobacter henricii* (96.8%) and *Caulobacter fusiformis* (96.8%) as its closest relatives. The non-prosthecate strain LMG 11050^T and the prosthecate strain FWC 21^T both belonged to the genus *Phenylobacterium* with *Phenylobacterium koreense* (96.9%) and *Phenylobacterium immobile* (96.3%) as the closest relatives. This affiliation was supported by chemotaxonomic data (polar lipids and cellular fatty acids). Physiological and biochemical tests allowed genotypic and phenotypic differentiation of the novel strains from all hitherto recognized species of the genera *Caulobacter* and *Phenylobacterium*. The strains therefore represent novel species, for which the names *Caulobacter mirabilis* sp. nov. (type strain FWC 38^T=LMG 24261^T=CCUG 55073^T), *Phenylobacterium conjunctum* (type strain FWC 21^T=LMG 24262^T=CCUG 55074^T), the first described prosthecate *Phenylobacterium* species, and *Phenylobacterium haematophilum* sp. nov. (type strain LMG 11050^T=CCUG 26751^T) are proposed. Marker nucleotides within the 16S rRNA genes were determined for the genera *Asticcacaulis*, *Brevundimonas*, *Caulobacter* and *Phenylobacterium* and the description of the genus *Phenylobacterium* is emended.

The genus *Caulobacter* was originally 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 cell is non-motile and prosthecate (Staley, 1968) and sticks to surfaces by adhesive material located at the tip of the prostheca. The other cell bears one polar flagellum by which it is motile. This mode of reproduction helps these oligotrophic bacteria to disperse the population at each generation, thereby minimizing competition for resources (Poindexter, 1981). *Caulobacteria* have been known for more than a century and the first isolation of a *Caulobacter* sp. was reported by Löffler in 1890 (Löffler, 1890). Even then, he noticed the unusual nutrient requirements of the strain due to the oligotrophic nature of the isolate. In 1935, Henrici and Johnson described the genus *Caulobacter* with *Caulobacter vibrioides* as the type species (Henrici & Johnson, 1935) but they did not isolate *C. vibrioides* and therefore no type strain was given. Poindexter (1964) proposed strain CB 51 as the neotype strain of *C. vibrioides* and described nine species and two

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Abbreviations: CID, collision-induced dissociation; FAB-MS, fast atom bombardment mass spectrometry; PG, phosphatidylglycerol; PGL, 3-O-[6'-(sn-glycero-3"-phosphoryl)-α-D-glucopyranosyl]-sn-glycerol.

Figures showing a phylogenetic tree constructed from 16S rRNA gene sequences using the minimal evolution algorithm and the negative fast atom bombardment mass spectra of the polar lipids are available as supplementary material with the online version of this paper. Tables detailing the cellular fatty acid contents, phospho- and sulfolipids, polar lipids, enzyme activities and the results of the Biolog tests for the species examined in this study are given in supplementary tables S1–S5, available with the online version of this paper.

subspecies (Poindexter, 1964, Poindexter & Lewis, 1966). By determining the 16S rRNA gene sequences of several isolates belonging to the genus *Caulobacter*, Stahl and coworkers revealed that the genus *Caulobacter* actually forms two different lineages, one comprising the freshwater and brackish water *Caulobacter* species and the other comprising the marine *Caulobacter* species (Stahl *et al.*, 1992) now harboured by the genus *Maricaulis* (Abraham *et al.*, 1999).

As caulobacteria are present in almost every water sample and are believed to play an important role in the carbon cycling within their habitats, the diversity of *Caulobacter* isolates was evaluated using lipid analysis, 16S rRNA gene sequences and profiling of the 16S-23S rRNA gene interspacer regions and these results were compared with physiological data. As a result, the descriptions of the genera *Caulobacter* and *Brevundimonas* were emended and a number of *Caulobacter* species were transferred to the genus *Brevundimonas* (Abraham *et al.* 1999). As part of an ongoing study, we identified a number of strains which could not be identified as any of the recognized species of caulobacteria. In this paper, three of the novel species that were identified from this study are described.

Strains for this study were obtained from the American Type Culture Collection (ATCC), the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSM), the Laboratorium voor Microbiologie, Universiteit Gent, Belgium, (LMG), The All-Russian Collection of Microorganisms (VKM) and from John Smit of the Department of Microbiology and Immunology of the University of British Columbia, Canada, (CB and FWC strains; Abraham *et al.*, 1999). All strains were grown in freshwater-Caulobacter-medium PYEM containing: 2 g peptone, 2 g yeast extract, 0.5 g NH_4Cl and 1 l Milli-Q water. After autoclaving and cooling, 5 ml riboflavin (0.2 mg ml^{-1}) sterile filtrated, 2 ml 50 % glucose (sterile), 1 ml 20 % MgSO_4 (sterile) and 1 ml 10 % CaCl_2 (sterile) were added. The strains were grown in 2 l flasks at 30 °C and 100 r.p.m. and the biomass was harvested in the late exponential phase after 72 h.

For electron microscopy, mid-exponential cells were adsorbed from an aliquot to Formvar-coated 300 mesh copper grids for 5 min at ambient temperature. Grids then were briefly blotted, washed for 15 s with deionized water and were either negatively stained with 1 % (w/v) uranyl acetate, pH 4.0, or air-dried and Pt-C shadow-cast at an angle of 10 degrees, according to Yakimov *et al.* (1998). For ultrathin section preparations, cells were fixed with 1.25 % (v/v) glutaraldehyde in the growth medium for 1 h at ambient temperature and were then stored at 4 °C for five days. Cells were washed twice in 100 mM cacodylate, pH 7.2, post-fixed with 1 % (w/v) osmium tetroxide/100 mM cacodylate, pH 7.2 for 60 min at ambient temperature and were then immobilized in 1 % (w/v) agar-water and dehydrated in an ascending ethanol series. The final ethanol phase was exchanged with acetone and

cells were embedded in hard grade epoxy resin (Spurr, 1969). Ultrathin sections (90 nm), negatively stained and Pt-C shadowed samples were analysed with an energy-filtered transmission electron microscope (EFTEM; CEM 902, Zeiss) at zero-loss (slit width: 20 eV; objective aperture: 30 μm) at 80 kV in a magnification range from $\times 7000$ to $\times 85000$. Micrographs were recorded with a 1024×1024 CCD slow scan camera (CCD HSS 512/1024; Proscan Electronic Systems).

Cells of strain FWC 21^T growing mid-exponentially were often organized as 'rosettes', formed by central clumping of the sticky holdfast termini (Fig. 1a). Characteristically, some prostheca showed predefined points of fissures (Fig. 1a: pf), which may help cells to dock off as mobile cells, though the general method of dispersal involves growth and diversion of polar, monotrichously flagellated daughter cells (Fig. 1a: sc). Residual prostheca are indicated (Fig. 1a: asterisks) as the remnants of offspring from the sessile state. Based on measurements of negatively stained swarming cells, cells of strain FWC 21^T were 1150–1590 nm in length and 540–670 nm in width. The cell wall was of a Gram-negative architecture and was about 25 nm in width (Fig. 1b: cytoplasmic membrane–outer membrane distance), layered with an S-layer (Fig. 1b: sl) of 7.4 nm thickness. Contrary to strain FWC 21^T, cells of strain LMG 11050^T did not show a stalk. As whole mount preparations, shadow-cast mid-exponential cells of strain FWC 21^T appeared as short rods, measuring 620–880 nm in width (mean: $718 \pm 70.6 \text{ nm}$; $n=24$) versus 950–1500 nm in length (mean: $1152 \pm 155 \text{ nm}$; $n=18$). Cells were flagellated and motile with a single flagellum, polarly inserted (Fig. 2a), which measured up to 7 μm in length. Apart from flagella and thin fimbriae, no further cellular appendices, such as prostheca, could be observed. A further characteristic feature of this strain was the presence of an S-layer, which could be recognized both in negatively stained (data not shown) and in Pt-C shadow-cast samples (Fig. 2c), showing a nearly orthogonal, almost square lattice and structural details down to 0.90 nm and 0.96 nm (Fig. 2b). When samples were embedded and ultrathin sections were examined (Fig. 2d), the cytoplasm was of general appearance, showing condensed chromosomal DNA within ribosomal dominated cytoplasmic matrix, which occasionally contained darkish inclusions (data not shown). At higher magnification, all the components of a Gram-negative cell wall structure were revealed.

Genomic DNA was isolated from a 2 ml sample of cultures 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. Appropriate amounts of DNA were enzymically digested and the mean guanine and cytosine (G+C) contents were determined by HPLC (Tamaoka & Komagata, 1984). Calculations were carried out according to Mesbah *et al.* (1989), with non-methylated λ -phage DNA (Sigma) used as a standard. DNA G+C contents of $67.0 \pm 0.1 \text{ mol\%}$ and $68.0 \pm 0.0 \text{ mol\%}$ were determined for strain FWC 21^T and

Fig. 1. Electron microscopy of cells of isolate FCW 21^T. (a) Negatively stained 'rosette' (pf, prosthecum fissure; sc, swarming cell; s, septum; *, residual prosthecum). (b) Ultrathin section of the cell wall (sl, S-layer; m, murein layer; om, outer membrane; cm, cytoplasmic membrane; cp, cytoplasm). Bars, 1 μ m (a) and 50 nm (b).

strain FWC 38^T, respectively. A value of 67.8 mol% has been reported previously for strain LMG 11050^T (Segers *et al.*, 1994). These values are at the upper limit of G+C values determined so far for species of the genera *Brevundimonas* and *Caulobacter* but they fit well with those reported for species of the genus *Phenylobacterium* (Aslam *et al.*, 2005).

For the amplification of the 16S rRNA gene by PCR, single colonies were boiled in 100 μ l of TE buffer for about 10 min at 95 °C. An almost complete 16S rRNA gene sequence was obtained as described previously (Abraham *et al.*, 1999). The reactions were evaluated on an Applied Biosystems 377 Genetic Analyzer and the final contig was assembled using the SEQUENCHER program, version 4.0.5 (Gene Codes Corporation). The sequence was matched in BLAST 2.2.9 (Altschul *et al.*, 1990) against the EMBL database (Kanz *et al.*, 2005). The 16S rRNA gene sequences of strains FWC 21^T, FWC 38^T and LMG 11050^T have been previously deposited at the EMBL database under the accession numbers AJ227767, AJ227774 and AJ244650, respectively. The sequences were aligned using CLUSTAL W software (Thompson *et al.*, 1997). Tree topologies were reconstructed with the UPGMA algorithm with 1000 bootstrap replications (Fig. 3) and the minimal evolution algorithm was calculated with MEGA 3.1 software (Kumar

et al., 2004) (see Supplementary Fig. S1 in IJSEM Online) using the EMBL database (Kanz *et al.*, 2005).

On the basis of 16S rRNA gene sequence similarity, strains FWC 21^T and LMG 11050^T were most closely related not to species of the genus *Caulobacter* but to *Phenylobacterium koreense* (96.95%) and to *Phenylobacterium immobile* (96.31%). Strain FWC 38^T was most closely related to *Caulobacter fusiformis* and *Caulobacter henricii* (both 96.80%). The 16S rRNA gene sequence of strain FWC 21^T showed 95.69% similarity to that of *C. fusiformis* ATCC 15257^T, 96.53% to *C. henricii* ATCC 15253^T, 95.83% to *C. vibrioides* VKM B-1496^T and 96.66% to FWC 38^T. The similarity between strains FWC 21^T and LMG 11050^T was 98.19%, but the two strains could easily be distinguished by their distinct morphology (Figs 1 and 2).

Because the species of the genera *Caulobacter* and *Phenylobacterium* are phylogenetically rather closely related, a search for nucleotides in the 16S rRNA gene sequences that might be useful to discern the genera of the order *Caulobacterales* was conducted. In this search, all type species of the genera *Caulobacter*, *Phenylobacterium*, *Brevundimonas* and *Asticcacaulis* were included. Nucleotides characteristic for one genus were checked against other strains clustering within the order

Fig. 2. Ultrastructure of cells of strain LMG 11050^T. (a) Individual, monopolar, monotrichously flagellated cell. Arrowhead indicates direction of shadow-casting. (b) Fast-Fourier Transform (FFT) of part of the surface layer, shown in (c), with ordered details down to 0.90 and 0.96 nm. (c) Shadow-cast bacterial surface, showing the presence of an S-layer. (d) Detailed view of the cell wall (90 nm ultrathin section), presenting the Gram-negative wall architecture. cm, Cytoplasmic membrane; pg, peptidoglycan; om, outer membrane; sl, surface layer; chr, bacterial chromosome. Bars, 1 μ m (a), 100 nm (c) and 50 nm (d).

Caulobacterales for consistency. The search revealed a number of nucleotides which could be used to identify strains within this order unambiguously (Table 1). All species were missing nucleotides 71–88, 183–190, 206–211 and 452–476 (*Escherichia coli* numbering). Nucleotides 1258–1278 which form loop 46 in V8 (Neefs *et al.*, 1993) were missing in the genera *Brevundimonas* and *Asticcacaulis*, but were present in the genera *Caulobacter* and *Phenylobacterium*. In this region, the genus *Phenylobacterium* had 1265-T and 1270-A while the genus *Caulobacter* had either 1265-C or -A and 1270-G or -T at these positions. All species of the genus *Phenylobacterium* had 122-G, while the other three genera in the order *Caulobacterales* had 122-A. Furthermore, species of the genus *Phenylobacterium* had 178-T, which was either G or A in the genera *Caulobacter*, *Brevundimonas* and *Asticcacaulis*. These three genera had 359-G but this was -A in all species of the genus *Phenylobacterium*. A marker nucleotide for the genus *Asticcacaulis* was 639-A, which was 639-G for all other members of the order *Caulobacterales*. Species of the genus *Brevundimonas* had 610-T, which was 610-G for the three other genera. Species of the genera *Brevundimonas* and *Asticcacaulis* had 823-A and 877-A, which were 823-G and 877-C for the genera *Caulobacter* and *Phenylobacterium*. Most species of the genus

Caulobacter had 1216-A, the only exception being strain FWC 38^T, which had 1216-G, similar to all species of the genera *Phenylobacterium*, *Brevundimonas* and *Asticcacaulis*.

The cellular fatty acid contents of the type strains of the genera *Caulobacter*, *Brevundimonas* and *Phenylobacterium* were determined by MIDI and are shown in Supplementary Table S1 (see IJSEM Online). Strain FWC 21^T possessed only trace amounts of C_{14:0} but considerable amounts of C_{12:1} 3-OH. It was the only strain in this study found to contain iso-C_{17:0}, although only in trace amounts, and the unidentified fatty acid ECL 17.897. Strain LMG 11050^T also contained only trace amounts of C_{14:0} and C_{17:1} ω 8c, but high amounts of summed feature 7 (see Supplementary Table S1). Strain FWC 38^T was exceptional in the large amounts of *trans*-11-methyl-12-octadecenoic acid (ECL 18.080) present, while the amounts of summed feature 7 (see Supplementary Table S1 in IJSEM Online) were considerably reduced. The *trans*-configuration of 11-methyl-12-octadecenoic acid was confirmed by ¹H-NMR spectroscopy in accordance to Albuquerque *et al.* (2002). C_{18:0}, a fatty acid found previously only in *C. henricii*, but lacking in all other *Caulobacter* strains (Abraham *et al.*, 1999), was detected in amounts below 1%. Strains FWC 21^T, FWC 38^T and LMG 11050^T did not contain ECL 11.798.

Fig. 3. Unrooted dendrogram using neighbour-joining based on the 16S rRNA gene sequences of strains FWC 38^T, FWC 21^T and LMG 11050^T and the type strains of the genera *Caulobacter*, *Phenylobacterium* and *Brevundimonas*. *Caulobacter crescentus* CB2^T is conspecific with *C. vibrioides* VKM 1496^T (Abraham *et al.*, 1999) and not shown here. Bar, 5 nucleotide substitutions per 1000 bases.

Strains of the genus *Caulobacter* were found to contain significant amounts of the fatty acids C_{12:1} 3-OH, C_{14:0}, C_{16:0}, summed feature 7 and ECL 18.080. All strains, except *Caulobacter segnis*, also contained C_{15:0}, C_{17:0}, C_{17:1}ω6c and C_{17:1}ω8c. Strains of the genus *Brevundimonas* could be differentiated from strains of the genus *Caulobacter* by the absence of ECL 11.789 and by the presence of at least trace amounts of ECL 17.897, only trace amounts of C_{12:1} 3-OH, higher amounts of C_{12:0} 3-OH and by a higher content of the dominant fatty acid, summed feature 7 (Abraham *et al.*, 1999). Species of the

genus *Phenylobacterium* differed from those of the genus *Caulobacter* by the lack of ECL 11.798, the presence of only trace amounts of C_{14:0} and a higher amount of C_{12:0} 3-OH and from the genus *Brevundimonas* by the presence of C_{12:1} 3-OH [most probably C_{12:1}ω6 according to Bellmann & Lingens (1985)], the occurrence of only trace amounts of C_{14:0} and the presence of C_{12:0}. Strains FWC 21^T, FWC 38^T and LMG 11050^T shared a lack of ECL 11.798 with species of the genus *Brevundimonas*. These strains shared the presence of considerable amounts of C_{12:1} 3-OH with species of the genus *Caulobacter* and the

Table 1. Marker nucleotides in the 16S rRNA genes of members of the order *Caulobacterales*

All species are lacking nucleotides 71–88, 183–190, 206–211, 452–476 (*E. coli* numbering). Nucleotides 1258–1278 are missing in the genera *Brevundimonas* and *Asticcacaulis* but are present in species of the genera *Caulobacter* and *Phenylobacterium*.

<i>E. coli</i> no.	<i>Phenylobacterium</i>	<i>Caulobacter</i>	<i>Brevundimonas</i>	<i>Asticcacaulis</i>
122	G	A	A	A
178	T (<i>falsum</i> : C)	G, A	G, A	G, A
359	A	G	G	G
610	G	G	T	G
639	G	G	G	A
823	G	G	A	A
877	C	C	A	A
1145	C	C	A	A
1265	T (<i>falsum</i> : A)	C, A	Missing	Missing
1270	A (<i>falsum</i> : T)	G, T	Missing	Missing

fatty acids of summed feature 7 were not as dominant as they were in *Brevundimonas* species. The unusually high amount of *trans*-11-methyl-12-octadecenoic acid in strain FWC 38^T resulted in a very distinctive lipid pattern as seen in the negative fast atom bombardment mass spectra of the polar lipids (see Supplementary Fig. S2 in IJSEM Online). The [M-H][−] ions at *m/z* 1410 or 1412 characteristic for strains of the genera *Caulobacter* or *Brevundimonas* were both absent and a very strong signal was observed for *m/z* 1438.

Lipids were extracted using a modified Bligh–Dyer procedure (Bligh & Dyer, 1959) as described previously (Vancanneyt *et al.*, 1996). The lipids were fractionated by sequential elution with dichloromethane, acetone and methanol, which resulted in three fractions of different polarity: neutral-, glyco- and phospholipids. The eluates were collected and dried under nitrogen. The phospholipid fractions were analysed by fast atom bombardment mass spectrometry (FAB-MS). FAB-MS in the negative mode was performed on the first of two mass spectrometers of a tandem high-resolution instrument in a E₁B₁E₂B₂ 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. A mixture of triethanolamine and tetramethylurea (Japanese matrix) was used as the matrix in the negative mode. 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 negative mode. The resolution of MS2 was set to 1:1000. FAB-CID spectra (linked scans of MS2 at constant B/E ratio) were recorded with 300 Hz filtering on a JEOL DA 7000 data system.

The polar lipids found in the novel strains examined in this study, together with those of the type strains of *Caulobacter* species, *Phenylobacterium immobile* DSM 1986^T, *Phenylobacterium falsum* LMG 22693^T and *Phenyl-*

bacterium lituiforme DSM 14363^T are given in Supplementary Table S2 (see IJSEM Online). All strains had phosphatidylglycerol (PG) and 3-O-[6'-(*sn*-glycero-3''-phosphoryl)- α -D-glucopyranosyl]-*sn*-glycerol (PGL) (Abraham *et al.*, 1997). The main PG for all of the strains was the 1-octadecenoyl-2-hexadecanoyl compound. Compared with strain FWC 38^T, strains FWC 21^T and LMG 11050^T had a larger variety of PG. The PGLs of these strains were not uniform. While strain FWC 21^T and LMG 11050^T possessed either PGLs of molecular mass 1411 or 1413, these lipids were completely absent in strain FWC 38^T. Instead, the major PGL was found at a molecular mass of 1439 due to the high content of *trans*-11-methyl-12-octadecenoic acid (11-methyl-C_{18:1} ω 5t, ECL 18.080). Only in strains LMG 11050^T and *P. falsum* LMG 22693^T could a sulfolipid belonging to the sulfoquinovosyl diacylglycerol type be detected (see Supplementary Table S2 in IJSEM Online). The analyses of the polar lipids of *Phenylobacterium immobile* DSM 1986^T and *Phenylobacterium lituiforme* DSM 14363^T led to the identification of 12 different PGs and 36 different PGLs, the majority of them described here for the first time (see Supplementary Table S3 in IJSEM Online). Only six of these 48 lipids were found in both species, underlining the fact that these are important biomarkers for the species. It was interesting to compare the PGLs of these two species of the genus *Phenylobacterium* with those identified in species of the genera *Caulobacter* and *Brevundimonas*. While all of the PGLs analysed from *Caulobacter* and *Brevundimonas* species so far have had C_{18:1} at the *sn*-1-position of the phosphatidyl moiety, this fatty acid is not always conserved in *Phenylobacterium* species and saturated fatty acids are also found at this position. Furthermore, species of the genera *Brevundimonas* and *Caulobacter* have either C_{18:1} or C_{19:1} at the *sn*-2'-position in the glycosyl moiety but this again is not conserved in members of the genus *Phenylobacterium*. PGLs with molecular masses <1400 have so far only been observed in *P. immobile* DSM 1986^T.

To determine the salt tolerances of the novel isolates, strains were grown in 20 ml PYEM medium amended with

0, 5, 10, 20, 30, 40, 60, 80 or 100 g l⁻¹ NaCl. The optical density of the cell suspension was determined at the beginning of the experiment and again after 2 days. The differences between these two measurements were used to determine salt tolerances. Substrate specificities were determined with the API Biotype 100 gallery (bioMérieux) and from Biolog GN substrate utilization tests (Rüger & Krambeck, 1994). Enzyme activities were assessed by API ZYM tests (bioMérieux) according to manufacturer's recommendations (see Supplementary Table S4). Strains FWC 21^T, FWC 38^T and LMG 11050^T could all grow without NaCl, but at a slightly reduced rate. They could also all grow at a concentration of 10 g l⁻¹ NaCl. Strain FWC 21^T showed good growth with 30 g l⁻¹ NaCl and could even grow with 40 g l⁻¹ NaCl, although only slowly. Strain FWC 38^T showed a similar salt tolerance, but growth with 40 g l⁻¹ NaCl was somewhat better than that of strain FWC 21^T. In contrast to these strains, strain LMG 11050^T could not grow in the presence of 20 g l⁻¹ NaCl.

Species of the genus *Caulobacter* show a characteristically small window of salt tolerances. They grow best with 5 g l⁻¹ NaCl and they do not tolerate concentrations of 20 g l⁻¹. In contrast, species of the genus *Brevundimonas* species grow best at concentrations of 0–30 g l⁻¹ NaCl and the upper limit of salt tolerance during growth varies widely between the different species. Strains FWC 21^T, FWC 38^T and LMG 11050^T all showed reduced growth without salt and at salt concentrations above 20 g l⁻¹, a characteristic similar to that found for species of the genus *Caulobacter*. Strain LMG 11050^T could not tolerate 20 g l⁻¹ salt, but strains FWC 21^T and FWC 38^T could grow slowly with 40 g l⁻¹ NaCl. Such a high salt tolerance has not been observed previously in the genus *Caulobacter*.

All of the novel strains could grow between 20 and 40 °C. The optimal growth temperatures were 30 °C for strain FWC 21^T, 40 °C for LMG 11050^T and 20–30 °C for strain FWC 38^T. While strains LMG 11050^T and FWC 38^T showed slow growth at 10 °C, no growth was seen for strain FWC 21^T.

As the species of the genus *Phenylobacterium* were more closely related to the genus *Caulobacter* than to the genus *Brevundimonas*, we attempted to use antibodies SMT2, specific for the genus *Caulobacter*, and SMT7, specific for the genus *Brevundimonas*, using the Westpriting technique (Abraham *et al.*, 1999). In our hands, these antibodies were not specific enough to differentiate species of the genus *Phenylobacterium* from those of the genera *Caulobacter* or *Brevundimonas*, although a slightly better reaction was observed for SMT2.

Conclusions

Due to the heterogeneity of the novel isolates, three different taxa could be discerned and a novel species of the genus *Caulobacter* and two novel species of the genus *Phenylobacterium* are proposed. The discriminating

characteristics of the newly proposed species and their nearest neighbours are summarized in Table 2 and Supplementary Table S5 (see IJSEM Online).

The inclusion of a prosthecate species into the genus *Phenylobacterium* requires an emendation of the genus description given by Lingens *et al.* (1985) and subsequently emended by Kanso & Patel (2004), Tiago *et al.* (2005) and Zhang *et al.* (2007).

Emendation of the genus *Phenylobacterium* (Lingens *et al.* 1985, emend. Kanso and Patel 2004, emend. Tiago *et al.* 2005, emend. Zhang *et al.* 2007)

Cells stain Gram-negative, are non-spore-forming straight to slightly curved rods, coccobacilli or cocci measuring 0.7–1.0 × 1.0–2.0 µm and occur singly, in pairs or short chains. Cells of some species have prosthecae. Cells may form rosettes. Filamentous cells tend to form in old cultures. Species may be strict aerobes or facultative anaerobes and may be motile or non-motile. Cells do not form sheaths and are not acid-fast. Species are lipase (C₁₄), α-galactosidase, α-mannosidase, β-glucosidase and α-fucosidase-negative. Based on the 16S rRNA gene sequence analysis, members of this genus form a monophyletic group within the order *Caulobacterales*, class *Alphaproteobacteria*. All species of the genus *Phenylobacterium* have 122-G in their 16S rRNA gene sequences while the other genera of the order *Caulobacterales* have 122-A. Furthermore, species of the genus *Phenylobacterium* have a 178-T which is either 178-G or 178-A in the genera *Caulobacter*, *Brevundimonas* and *Asticcacaulis*. These three genera have a 359-G which is 359-A in all species of the genus *Phenylobacterium*. Main cellular fatty acids are C_{16:0} and C_{18:1ω7}. In contrast with species of the genera *Brevundimonas* and *Caulobacter*, C_{14:0} is present only in traces. Hydroxy fatty acids are C_{12:0} 3-OH and C_{12:1} 3-OH. All species of the genus *Phenylobacterium* have the phospholipids phosphatidylglycerol and 3-O-[6'-(sn-glycero-3"-phosphoryl)-α-D-glucopyranosyl]-sn-glycerols. Some species also possess the sulfolipids sulfoquinovosyl diacylglycerols. The DNA G + C ratio is between 66.5 and 68.5 mol%. Isolated from soil, water and blood. The type species is *Phenylobacterium immobile* Lingens *et al.* 1985.

Description of *Phenylobacterium conjunctum* sp. nov.

Phenylobacterium conjunctum (con.junc'tum. L. neut. adj. *conjunctum* adjacent, the *Phenylobacterium* species which is closely related to the genus *Caulobacter*).

The description of *Phenylobacterium conjunctum* is the same as that given for the genus (Lingens *et al.*, 1985) and emended by Kanso & Patel (2004) and in this study, with the following additional characteristics. Prosthecate bacteria, colony colour is tan, cells are bacteroid. Cells are

Table 2. Discriminating characteristics of species of the genera *Caulobacter* and *Phenylobacterium*

Taxa, 1, strain FWC 38^T; 2, *C. vibrioides*; 3, *C. henricii*; 4, *C. segnis* (data from Urakami *et al.*, 1990); 5, strain FWC 21^T; 6, *P. immobile* DSM 1986^T (Aslam *et al.*, 2005); 7, *P. lituiforme* DSM 14363^T (Aslam *et al.*, 2005); 8, *P. koreense* Slu-01^T (Aslam *et al.*, 2005); 9, *P. falsum* AC-49^T (Tiago *et al.*, 2005); 10, '*Phenylobacterium zucineum*' (Zhang *et al.*, 2007); 11, strain LMG 11050^T (Segers *et al.*, 1994). +, Positive; (+), weakly positive; –, negative; ND, not determined.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
DNA G + C (mol%)	68.0	ND	ND	67–68	67.0	67.8	66.5*	68.1	66.9	71.2	67.8
Prostheca	+	+	+	–	+	–	–	–	–	–	–
Colour	Yellow	Colourless	Yellow	Colourless	Tan	Colourless	Yellow	Colourless	White	Grey	Whitish
NaCl tolerance (g l ^{–1})	40	<10	10	ND	<20	<10*	<10	20	70	<60	10
Oxidase	+	ND	ND	+	+	–	–	+	+	+	+
Nitrate reduction	–	ND	ND	–	–	–*	+	+	–	+	–
N ₂ from NO ₃ [–]	–	ND	ND	–	–	–	+	–	–	ND	–
Utilization of:											
Acetate	–	–	–	–	–	–	+	+	ND	–	–
Citrate	–	–	–	–	+	–*	+	–	–	–	+
Fumarate	–	–	–	–	ND	(+)	+	–	ND	ND	ND
Glucose	+	–	–	–	+	–*	+	–	–	+	+
Glycerol	–	–	–	–	–	–	+	–	–	ND	–
Phenylalanine	+	–	–	–	–	+	+	+	–	+	–
Succinate	+	–	–	–	–	(+)*	+	–	+	ND	–
Sucrose	–	–	–	–	–	–*	+	–	–	–	–
Xylose	–	–	–	–	ND	–*	+	–	–	ND	–
Malonate	–	–	–	–	+	–	–	–	ND	–	–
α-L-Rhamnose	+	–	–	–	–	–	–	–	–	–	–
N-Acetylglucosamine	+	–	–	–	–	–	–	–	ND	–	–
DL-Lactate	+	–	–	–	–	–	–	–	ND	–	–
β-Hydroxybutyrate	+	–	–	–	–	–	–	+	–	+	–
L-Aspartate	+	–	–	–	–	–	–	ND	–	ND	–
L-Glutamate	+	–	–	–	–	–	–	ND	+	ND	–
L-Proline	+	–	–	–	–	–	–	+	+	+	–
L-Alanine	(+)	–	–	–	–	–	–	–	+	–	–

*Data taken from Tiago *et al.* (2005).

1.15–1.60 µm in length and 0.5–0.7 µm in width. The cell wall is of Gram-negative architecture and is about 25 nm in width, layered with an S-layer of 7.4 nm thickness. Two major fatty acids are summed feature 7 (see Supplementary Table S1) and C_{16:0}. The minor fatty acids are summed feature 4 (see Supplementary Table S1) and C_{15:0}. Can grow on peptone-yeast extract media with 0–10 g l^{–1} NaCl, with optimal growth at around 5 g l^{–1} NaCl. Tests are positive for the activities of acid and alkaline phosphatase, esterase (C₄), leucine arylamidase, naphthol-AS-BI-phosphohydrolase and weak activities are detected for cystine arylamidase, esterase lipase (C₈), trypsin, N-acetyl-β-glucosaminidase, α-glucosidase and α-chymotrypsin. No activity for α-fucosidase, α- or β-galactosidase, α-glucuronidase, β-glucosidase, α-mannosidase or valine arylamidase. Able to utilize arabinose, citrate, dextrin, glucose, malonate, maltose, mannose and Tween 40, but not acetate, N-acetyl-D-glucosamine, cis-aconitic acid, adonitol, alaninamide, L- and D-alanine, L-alanylglycine, 2-amino ethanol, γ-aminobutyric acid, D-arabitol,

L-asparagine, L-aspartic acid, bromosuccinic acid, 2,3-butanediol, DL-carnitine, cellobiose, α-cyclodextrin, i-erythritol, formate, D-fructose, L-fucose, D-galactose, D-galactonic acid lactone, D-galacturonic acid, gentiobiose, D-gluconic acid, D-glucosaminic acid, glucose 1- and glucose 6-phosphate, glucuronamide, D-glucuronic acid, L-glutamic acid, glycerol, DL-α-glycerol phosphate, glycogen, glycyl-L-glutamic acid, L-histidine, α-, β- and γ-hydroxybutyric acids, p-hydroxyphenylacetic acid, hydroxy L-proline, inosine, myo-inositol, itaconic acid, α-ketobutyrate, α-ketoglutarate, α-ketovaleric acid, DL-lactate, α-D-lactose, lactulose, L-leucine, D-mannitol, melibiose, methyl β-D-glucoside, glycyl L-aspartic acid, methyl pyruvate, monomethyl succinate, L-ornithine, L-phenylalanine, phenylethylamine, L-proline, propionate, psicose, putrescine, L-pyroglutamic acid, quinic acid, raffinose, L-rhamnose, D-saccharic acid, sebacic acid, D- and L-serine, D-sorbitol, succinamic acid, succinic acid, sucrose, Tween 80, trehalose, L-threonine, turanose, thymidine, uridine, urocanic acid or xylitol. Does not grow with salt

concentrations above 20 g l⁻¹ NaCl. Growth is observed between 20 and 40 °C, with optimal growth at around 30 °C. The DNA G+C content is 67.0 mol%.

The type strain, FWC 21^T (=LMG 24262^T=CCUG 55074^T), was isolated from a bench-scale rotating biological contactor at the University of British Columbia, Canada.

Description of *Phenylobacterium haematophilum* sp. nov.

Phenylobacterium haematophilum (hae.ma.to.phi'lum. Gr. n. *haema* -atos blood; Gr. adj. *philos* loving; N.L. neut. adj. *haematophilum* blood-loving).

The description of *Phenylobacterium haematophilum* is the same as that given for the genus (Lingens *et al.*, 1985) and emended by Kanso & Patel (2004) and in this study, with the following additional characteristics. The polar lipid fraction contains phosphatidylglycerols, sulfoquinovosyl diacylglycerol of 834 amu and 6-phosphatidyl- α -D-glucopyranosyl diacylglycerol of 1413 amu or higher. Major fatty acids are C_{18:1} ω 7 and C_{16:0} (see Supplementary Table S1), minor fatty acids are summed feature 4, C_{15:0} and *trans*-11-methyl-12-octadecenoic acid. The main hydroxyl fatty acid is C_{12:0} 3-OH (see Supplementary Table S1). Is able to grow on peptone-yeast extract media with 0–10 g l⁻¹ NaCl with optimal growth at around 5 g l⁻¹ NaCl. Does not tolerate salt concentrations above 20 g l⁻¹ NaCl. Growth is observed between 10 and 40 °C, with optimal growth around 37 °C. Positive in results for activities of acid and alkaline phosphatase, esterase (C₄), leucine arylamidase, naphthol-AS-BI-phosphohydrolase and for weak activities of cystine arylamidase, esterase lipase (C₈), trypsin, *N*-acetyl- β -glucosaminidase, α -glucosidase and α -chymotrypsin. No activity is detected for α -fucosidase, α - and β -galactosidase, α -glucuronidase, β -glucosidase, α -mannosidase and valine arylamidase. Is able to utilize arabinose, citrate, β -hydroxybutyric acid, α -D-glucose, L-glutamic acid, glycyl-L-glutamic acid, itaconic acid, maltose, mannose, methyl β -D-glucoside, methyl pyruvate, L-proline, propionate, sebacic acid and Tweens 40 and 80. Does not utilize acetate, *N*-acetyl-D-glucosamine, *cis*-aconitic acid, adonitol, alaninamide, L- and D-alanine, L-alanyl glycine, γ -aminobutyric acid, 2-aminoethanol, D-arabitol, L-asparagine, L-aspartic acid, bromosuccinic acid, 2,3-butanediol, DL-carnitine, cellobiose, α -cyclodextrin, dextrin, i-erythritol, formate, D-fructose, L-fucose, D-galactonic acid lactone, D-galactose, D-galacturonic acid, gentiobiose, D-glucuronic acid, D-glucosaminic acid, glucose 1- and 6-phosphate, glucuronamide, D-glucuronic acid, glycerol, DL- α -glycerol-phosphate, glycogen, glycyl-L-aspartic acid, L-histidine, α -hydroxybutyric acid, γ -hydroxybutyric acid, *p*-hydroxyphenylacetic acid, hydroxy L-proline, inosine, *myo*-inositol, α -ketobutyric acid, α -ketoglutaric acid, α -ketovaleric acid, DL-lactate, α -lactose, L-leucine, malonate, D-mannitol, melibiose, monomethylsuccinate, L-ornithine, L-phenylalanine, phenylethylamine, psicose, putrescine, L-pyroglytamic acid, quinic acid, raffinose, L-rhamnose, D-saccharic

acid, D- and L-serine, D-sorbitol, succinamic acid, succinate, sucrose, L-threonine, thymidine, trehalose, turanose, uridine, urocanic acid and xylitol. The species can use adonitol but not (+)-L-arabinose, caprate, β -(+)-D-fructose, (+)-D-galactose, α -(+)-D-glucose, glycerol, L-histidine, *myo*-inositol, α -lactose, maltose, D-mannitol, (+)-D-mannose, D-sorbitol, sucrose, (+)-D-trehalose or (+)-D-xylose. The G+C content is 67.8 mol%.

The type strain, LMG 11050^T (=CCUG 26751^T), was isolated from human blood (Göteborg, Sweden).

Description of *Caulobacter mirabilis* sp. nov.

Caulobacter mirabilis (mi.ra'bi.lis. L. masc. adj. *mirabilis* strange, the strange *Caulobacter* which is unique in its high content of *trans*-11-methyl-12-octadecenoic acid).

The description of *Caulobacter mirabilis* is the same as that given for the genus (Henrici & Johnson, 1935, emended by Poindexter, 1964; Abraham *et al.*, 1999), with the following additional characteristics. Prosthecate bacteria, colony colour is bright yellow, cells are bacteroid. The two major fatty acids are *trans*-11-methyl-12-octadecenoic acid and C_{16:0}. Minor fatty acids are summed feature 7 (see Supplementary Table S1), C_{15:0}, C_{17:0}, and C_{20:0}. The 6-phosphatidyl- α -D-glucopyranosyl diacylglycerol fraction does not contain the compounds of 1409 amu, characteristic for the *Caulobacter* species or 1411 amu, but does contain compounds of 1439 and 1453 amu (Abraham *et al.*, 1997). Positive in tests for the activities of alkaline and acid phosphatase, esterase (C₄), esterase lipase (C₈), leucine arylamidase, naphthol-AS-BI-phosphohydrolase and trypsin. Weak activities for cystine arylamidase and α - and β -glucosidase and no activities for α -chymotrypsin, α -fucosidase, α - and β -galactosidase, α -glucuronidase, lipase (C₁₄), *N*-acetyl- β -glucosaminidase, α -mannosidase or valine arylamidase. Can utilize *N*-acetyl-D-glucosamine, D- and L-alanine, asparagine, L-aspartate, aesculin, α -(+)-D-glucose, L-glutamate, 3-hydroxybutyrate, DL-lactate, maltose, L-proline, pyruvate, α -L-rhamnose, succinate, L-serine and Tween 80, but not *cis*- or *trans*-aconitate, adonitol, 4-aminobutyrate, 5-aminovalerate, (+)-L-arabinose, (+)-D- or (-)-L-arabitol, benzoate, betain, caprate, caprylate, *m*-coumarate, (+)-D-cellobiose, citrate, dulcitol, i-erythritol, ethanolamine, α -(+)-D-fructose, α -L-fucose, fumarate, (+)-D-galactose, D-galacturonate, β -(+)-D-gentiobiose, gentisate, D-gluconate, D-glucosamine, D-glucuronate, glutarate, DL-glycerate, glycerol, histamine, L-histidine, hydroxyquinoline- β -glucuronide, 3- or 4-hydroxybenzoate, *myo*-inositol, itaconate, 2- or 5-keto-D-glucuronate, 2-ketoglutarate, α -lactose, lactulose, D-lyxose, malonate, maltitol, D-mannitol, (+)-D-mannose, (+)-D- or (-)-L-malate, maltotriose, (+)-D-melezitose, α -(+)-D-melibiose, 1-O-methyl- α - and β -galactopyranoside, 1-O-methyl α - and β -D-glucopyranoside, 3-O-methyl D-glucopyranoside, mucate, palatinose, phenylacetate, 3-phenylpropionate, propionate, protococatechuate, putrescine, quinate, (+)-D-raffinose, (-)-D-ribose, D-saccharate, D-

sorbitol, (+)-L-sorbose, sucrose, D-tagatose, (+)-L-, (-)-D- or *meso*-tartrate, (+)-D-trehalose, tricarallylate, trigonelline, tryptamine, L-tryptophan, L-tyrosine, (+)-D-turanose, xylitol or (+)-D-xylose. The strain can grow on blood agar or on peptone-yeast extract media with 0–40 g l⁻¹ NaCl with optimal growth between 5 and 20 g l⁻¹ NaCl. Does not tolerate salt concentrations above 60 g l⁻¹ NaCl. Growth occurs between 10 and 40 °C, with optimal growth between 20 and 30 °C. No S layer, but multiple bands of lipopolysaccharides are detected (Walker *et al.*, 1992). The DNA G+C content is 68.0 mol%.

The type strain, FWC 38^T (=CCUG 55073^T=LMG 24261^T), was isolated from the activated sludge system of a secondary treatment facility, Las Vegas, Nevada, USA.

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