Reclassification of the polyphyletic genus *Prosthecomicrobium* to form two novel genera, *Vasilyevaea* gen. nov. and *Bauldia* gen. nov. with four new combinations: *Vasilyevaea enhydra* comb. nov., *Vasilyevaea mishustinii* comb. nov., *Bauldia consociata* comb. nov. and *Bauldia litoralis* comb. nov.

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Species of the genus *Prosthecomicrobium* are noted for their numerous cellular appendages or prosthecae that extend from the cells. This investigation confirms that the genus is polyphyletic based on an extensive analysis of the 16S rRNA gene sequences of several named species of the genus. The analyses indicate that some *Prosthecomicrobium* species are more closely related to non-prosthecae genera, including *Devosia*, *Labrenzia*, *Blastochloris*, *Methylosinus*, *Mesorhizobium* and *Kaistia*, than they are to other species of the genus *Prosthecomicrobium*. For this reason, two of the *Prosthecomicrobium* clades which are polyphyletic with the type species, *Prosthecomicrobium pneumaticum*, are renamed as new genera. The currently named species *Prosthecomicrobium enhydrum*, *Prosthecomicrobium mishustinii*, *Prosthecomicrobium consociatum* and *Prosthecomicrobium litoralis* are reclassified in two new genera, *Vasilyevaea* gen. nov. and *Bauldia* gen. nov. with four new combinations: *Vasilyevaea enhydra* comb. nov. (the type species) and *Vasilyevaea mishustinii* comb. nov., and *Bauldia consociata* comb. nov. and *Bauldia litoralis* comb. nov. (the type species). The type strain of *Vasilyevaea enhydra* is strain 9b^T (=ATCC 23634^T =VKM B-1376^T). The type strain of the other species in this genus is *Vasilyevaea mishustinii* strain 17^T (=VKM B-2499^T =CCM 7569^T). The type strain of *Bauldia litoralis* is strain 524-16^T (=NCIB 2233^T =ATCC 35022^T). The type strain of the other species in this genus is *Bauldia consociata* strain 11^T (=VKM B-2498^T =CCM 7594^T).

Bacteria of the genus *Prosthecomicrobium*, which is a member of the class *Alphaproteobacteria*, reproduce by budding and produce several cellular appendages or prosthecae that extend from each cell in all directions. They were first described from isolates obtained from fresh water (Staley, 1968). Subsequently, other species were reported that were isolated from various other habitats including soil (Vasil'eva *et al.*, 1974), pulp mill aeration ponds, and brackish and marine water (Stanley *et al.*, 1979; Bauld *et al.*, 1983; Schlesner *et al.*, 1989). Species with validly published names include *Prosthecomicrobium pneumaticum*, *P. enhydrum* (Staley, 1968), *P. litoralis* (Bauld *et al.*, 1983), *P. hirschii* (Staley, 1984), *P. mishustinii* and *P.*

consociatum (Lafitskaya *et al.*, 1976; Vasil'eva *et al.*, 1991). The names of the latter two species were recently validly published (Vasil'eva *et al.*, 2009).

The primary phenotypic, differentiating features among species of the genus *Prosthecomicrobium* are morphological, in particular the number and length of the prosthecae and whether or not cells are motile. Other differentiating features among species of these aerobic bacteria are colony pigmentation, carbon source utilization and presence of gas vesicles.

Phylogenetic analyses of 16S rRNA genes of strains representing species of the genus *Prosthecomicrobium* have revealed the polyphyletic nature of the group, which forms several independent clusters within the class *Alphaproteobacteria* (Schlesner *et al.*, 1989; Oertli *et al.*, 2006). Furthermore, chemotaxonomic analysis of fatty

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences determined in this study are FJ560749, FJ560750 and GQ221761–GQ221768.

acids, in which three identified type species were included, also indicated that at least five distinct subgroups exist within the current genus *Prosthecomicrobium* (Sittig & Schlesner, 1993). The reason for the polyphyly is not understood; however, two explanations are possible. First, the genes that are responsible for the formation of prosthecae may be ancestral to the family that contains these bacteria. Then, through evolution, some of the genera and species lost these genes whereas others retained them. Alternatively, one could postulate that genetic exchange has occurred in which the genes responsible for prosthecae formation were transferred from one prosthecae species to another closely related, non-prosthecae species through horizontal gene transfer. Perhaps the genome sequences of several representative prosthecae and non-prosthecae species in this group will aid in resolving this issue.

Until now, no attempt has been made to reclassify the genus *Prosthecomicrobium* to reflect its polyphyletic nature. In this paper, we report the results we have obtained from further comparative phenotypic and phylogenetic analyses. Based on these findings, we propose the reclassification of several species of the genus *Prosthecomicrobium* into two new genera.

16S rRNA gene reference sequences were selected from representatives of different orders within the class *Alphaproteobacteria*. These were then compared to 16S rRNA gene sequences of the various species of the genus *Prosthecomicrobium* including all type strains (Oertli *et al.*, 2006) as well as others from the RNA database (Cole *et al.*, 2003). The sequence match tool within the RDP-II website was used to identify the sequences for alignment based on best matches. Sequence alignment for phylogenetic tree reconstruction was performed using the NAST (Nearest Alignment Space Termination; DeSantis *et al.*, 2006) function available at the Greengenes website (<http://greengenes.lbl.gov>) (DeSantis *et al.*, 2006) and the alignment was also subjected to Lane masking (Lane, 1991) of ambiguous sites using the column masking tool available at the Greengenes website.

Phylogenetic trees were reconstructed using TreeFinder (Jobb *et al.*, 2004) by applying the substitution model of GTR+I+G, the optimal substitution model selected by Modelgenerator (Keane *et al.*, 2006). A maximum-likelihood tree was reconstructed using TreeFinder (Jobb *et al.*, 2004). Distance and maximum-parsimony trees were reconstructed using the PHYLIP package (Felsenstein, 2007). Bootstrap analysis was performed using 1000 replicates for all trees. Similarity values were calculated using PHYLIP.

The 16S rRNA gene sequence tree of the genus *Prosthecomicrobium* and those genera and species to which it is most closely related illustrates its polyphyletic nature (Fig. 1). It is noteworthy that the type species of the genus, *Prosthecomicrobium pneumaticum*, the only known gas vacuolate species in the genus, is clustered with only one other strain, *Prosthecomicrobium* sp. ATCC 27835, to which it has 99% sequence similarity. These two strains are

separated from the other named species of the genus *Prosthecomicrobium* by the genera *Methylosinus*, *Blastochloris* and *Kaistia* within the class *Alphaproteobacteria*. Therefore, *P. pneumaticum* is more closely related to the methane-oxidizing bacteria *Methylosinus trichosporium* and *Methylocystis echinoides* (Whittenbury *et al.*, 1970) and the phototrophic bacterial genus *Blastochloris* (Keppen & Gorlenko, 1975; Hiraishi, 1997) than it is to other species of the genus *Prosthecomicrobium*.

There is strong bootstrap support in Fig. 1 (>75% at the primary and secondary nodes of the trees) for the clustering of the other species of the genus *Prosthecomicrobium* with other genera and separate from the type species *P. pneumaticum*. Therefore, this is the basis for the reclassification of several described species of the genus *Prosthecomicrobium* into new genera.

Consider the described species *P. enhydrum* and *P. mishustinii* that form an isolated group with *Devosia neptuniae* (Fig. 1). The phylogenetic difference between this cluster and *P. pneumaticum* is illustrated by a comparison of *P. pneumaticum* and *P. enhydrum*, which share only 93.3% 16S rRNA gene sequence similarity (data not shown). Furthermore, these two species have different phospholipids and fatty acids (Sittig & Schlesner, 1993). For example, the phospholipids of *P. pneumaticum* include large amounts of phosphatidylglycerol, phosphatidylethanolamine and phosphatidylidimethylethanolamine with lower amounts of bisphosphatidylglycerol whereas *P. enhydrum* contains only phosphatidylglycerol and bisphosphatidylglycerol (Sittig & Schlesner, 1993). Since *P. enhydrum* and *P. mishustinii* are clustered independently of *P. pneumaticum*, we propose a new genus, *Vasilyevaea* gen. nov., with two species, *Vasilyevaea enhydra* comb. nov. and *Vasilyevaea mishustinii* comb. nov., for this novel cluster. It should be noted that although these two species are quite closely related to one another with 98.9% 16S rRNA gene sequence similarity, they are separate species based upon DNA hybridization analyses (Vasil'eva *et al.*, 1991).

Similarly, *P. litoralum* and *P. consociatum* (with 97.1% 16S rRNA gene sequence similarity between them) form a clade that is separate from the type species *P. pneumaticum*, with which they have only 93.7% and 94.4% rRNA gene sequence similarity, respectively, as well as from *Vasilyevaea enhydra* comb. nov. and other clusters containing members of the genus *Prosthecomicrobium*. A new genus is therefore proposed for this group of prosthecae bacteria, *Bauldia* gen. nov., with two species, *Bauldia litoralis* comb. nov. and *Bauldia consociata* comb. nov.

Prosthecomicrobium hirschii strains also cluster in a separate clade away from *P. pneumaticum* and other species of the genus *Prosthecomicrobium*. This prosthecae species has a life cycle with two different morphological stages that distinguishes it from all other *Prosthecomicrobium* species (Staley, 1984). This species should also be reclassified in a new genus (G. E. Oertli & J. T. Staley, unpublished).

Fig. 1. Maximum-likelihood tree based on 16S rRNA gene sequences showing the relationship of the *Prosthecomicrobium* strains (in bold) and the new genera within the class Alphaproteobacteria. The *Escherichia coli* 16S rRNA gene sequence was used as an outgroup to root the tree. Circles indicate consensus bootstrap values from maximum-likelihood, distance and maximum-parsimony analyses. Nodes supported at $\geq 75\%$ in the majority of analyses are indicated by filled circles. Nodes supported at 50–74% in most analyses are indicated by open circles. Unsupported nodes ($\leq 50\%$) have no circle. Bar, 0.1 substitutions per site.

In addition to prostheca formation, another major feature that distinguishes the genus *Prosthecomicrobium* as well as the two newly proposed genera, *Vasilyevaea* gen. nov. and *Bauldia* gen. nov., from the other non-prosthecate genera is reproduction by budding (Table 1). Also, some of the non-prosthecate genera that are closely related to the genera *Prosthecomicrobium*, *Vasilyevaea* gen. nov. and *Bauldia* gen. nov. are phototrophs (*Blastochloris*) whereas others are methanotrophs (*Methylocystis* and *Methylosinus*), nitrogen fixers (*Mesorhizobium*) or are simply non-budding, non-prosthecate heterotrophic bacteria (*Kaistia*, *Devosia* and *Labrenzia*) (Table 2).

Species of the genera *Vasilyevaea* gen. nov. and *Bauldia* gen. nov. can be differentiated from *Prosthecomicrobium pneumaticum* by their lack of gas vesicles. Species of the genus *Bauldia* gen. nov. differ from those of the genus *Vasilyevaea* gen. nov. and from *Prosthecomicrobium pneumaticum* by their ability to use methanol as a sole carbon source. Colonies of species belonging to the genus

Vasilyevaea are typically pigmented whereas those of *Prosthecomicrobium pneumaticum* and species of the genus *Bauldia* are white or grey (Table 2). Individual species within the two new genera can be readily differentiated on the basis of phenotypic properties such as agar digestion, motility, fatty acid composition and carbon source utilization (Tables 2 and 3).

Therefore, both phylogenetic and phenotypic properties of this group of closely related genera support the proposed reclassification. A description of the two new genera follows.

Description of *Vasilyevaea* gen. nov.

Vasilyevaea (Va.sil'ye.vae.a. N.L. fem. n. *Vasilyevaea* of Vasilyeva, named in honour of Lina Vasilyeva, a Russian microbiologist who has dedicated her career to the investigation of prosthecate bacteria and has named several new genera within this group).

Table 1. Differentiation of the genus *Prosthecomicrobium* from closely related genera

Genera: 1, *Prosthecomicrobium**; 2, *Vasilyevaea* gen. nov.; 3, *Bauldia* gen. nov.; 4, *Devosia*; 5, *Labrenzia*; 6, *Methylosinus* and *Methylocystis*; 7, *Blastochloris*; 8, *Mesorhizobium*; 9, *Kaistia*. +, Positive; –, negative.

Characteristic	1	2	3	4	5	6	7	8	9
Prosthecae	+	+	+	–	–	–	–	–	–
Budding division	+	+	+	–	–	–	–	–	–
Gas vesicles	+	–	–	–	–	–	–	–	–
Phototrophic	–	–	–	–	–	–	+	–	–
Methanotrophic	–	–	–	–	–	+	–	–	–
Nitrogen fixation	–	–	–	–	–	–	–	+	–
DNA G + C content (mol%)	69–70	63–66	66–69	61–62	56–60	61–67	66–72	59–64	67–68

*Features listed are for the type species of the genus *Prosthecomicrobium* only.

Gram-negative, budding, prosthecae bacteria with numerous short appendages, less than 1 µm in length, that cover the cell surface. Aerobic and heterotrophic. Use

a variety of carbon sources including sugars and sugar alcohols for growth. Cells may be pigmented. Organisms are found in fresh water or soils. DNA G + C content

Table 2. Selected characteristics of *Vasilyevaea enhydra* comb. nov. and *V. mishustinii* comb. nov. in comparison with the closely related species *Devosia neptuniae* and *Mesorhizobium mediterraneum*

Species: 1, *Vasilyevaea enhydra* comb. nov. (data from this study; fatty acid data from Sittig & Schlesner, 1993); 2, *V. mishustinii* comb. nov. (this study); 3, *D. neptuniae* (Rivas *et al.*, 2003; fatty acid data from Yoon *et al.*, 2007); 4, *M. mediterraneum* (Nour *et al.*, 1994; fatty acid data from Tighe *et al.*, 2000). Universal fatty acid nomenclature is used; data not provided if less than 0.2% for a known fatty acid. +, Positive; –, negative; ±, indefinite; ND, no data available; NA, not applicable.

Characteristic	1	2	3	4
Cell shape	Irregular	Thick rods	Rods	Rods
Presence of prosthecae	+	+	–	–
Prosthecae length (µm)	0.5	<0.65	NA	NA
Motility	+	–	+	+
Gas vacuoles	–	–	–	–
Carbon source				
D-Glucose	+	+	+	+
Maltose	+	+	+	+
Lactose	+	+	ND	ND
Sorbitol	–	–	ND	+
Mannitol	–	+	+	+
Malate	+	+	–	+
Pyruvate	+	±	ND	+
Methanol	–	–	ND	ND
Non-hydroxy fatty acids (%)				
C _{16:0}	15.1	ND	25.6	10.3
C _{17:0}	0.3	ND	–	1.7
iso-C _{17:0}	–	ND	–	4.2
C _{17:0} cyclo	–	ND	2.6	0.9
C _{18:0}	5.0	ND	6.7	4.2
C _{18:1ω7c}	50.5	ND	21.6	36*
11-Methyl C _{18:1ω6}	26.9	ND	–	–
11-Methyl C _{18:1ω7c}	–	ND	–	5.6
C _{19:0} cyclo ω8c	–	ND	7.8	33.4
DNA G + C content (mol%)	66	64–65	62–62.4	63–64
Colony colour	White; yellow; red	Yellow–orange	Pearl white	Opaque

*Summed feature 7 of Tighe *et al.* (2000) that contains C_{18:1ω7c/ω9t/ω12t} and/or C_{18:1ω7c/ω9c/ω12t}.

Table 3. Selected characteristics of *Bauldia litoralis* comb. nov. and *Bauldia consociata* comb. nov. in comparison with those of closely related species

Species: 1, *Bauldia litoralis* comb. nov.; 2, *Bauldia consociata* comb. nov.; 3, *Prosthecomicrobium pneumaticum*; 4, *Kaistia adipata* (data from Im *et al.*, 2004); 5, *Blastochloris sulfovirens* (Keppen and Gorlenko, 1975); 6, *Methylosinus trichosporium* (Whittenbury *et al.*, 1970). +, Positive; –, negative; ±, indefinite; ND, no data available; NA, not applicable.

Characteristic	1	2	3	4	5	6
Cell shape	Rods	Rods	Rods	Rods	Ovoid	Rods
Presence of prosthecae	+	+	+	–	–	–
Prostheca length (µm)	<1	<0.25	<1	NA	NA	NA
Motility	–	–	–	–	+	+
Exospores	–	–	–	–	–	+
Gas vacuoles	–	–	+	–	–	–
Carbon source						
D-Glucose	+	+	+	+	+	–
Maltose	+	–	+	+	+	–
Lactose	+	–	+	+	ND	–
Mannitol	+	+	+	+	–	ND
Sorbitol	+	–	+	+	+	–
Malate	+	–	–	–	+	–
Propionate	–	–	–	–	–	ND
Pyruvate	+	–	–	ND	–	–
Methanol	+	±	–	ND	–	+*
Methane	ND	ND	ND	ND	ND	+
Agar digestion	+	–	–	–	–	–
DNA G + C content (mol%)	66–67	66–69	69–70	67.4	67.8–68.4	62–63
Colony colour	White	Grey	White	Ivory	Olive green	White–Yellow

*Inhibited by high concentrations.

(mol%) ranges from 63 to 66. The type species is *Vasilyevaea enhydra*.

Description of *Vasilyevaea enhydra* comb. nov.

Vasilyevaea enhydra (en.hy'dra N.L. adj. *enhya* living in water, aquatic).

Basonym: *Prosthecomicrobium enhydrum* Staley 1968.

Characteristics are as described by Staley (1968) and Oertli *et al.* (2006). In addition, this species contains phosphatidylglycerol and bisphosphatidylglycerol (Sittig & Schlesner, 1993). The DNA G + C content of the type strain is 65.8 mol% (buoyant density).

The type strain is strain 9b^T (=ATCC 23634^T =VKM B-1376^T).

Description of *Vasilyevaea mishustinii* comb. nov.

Vasilyevaea mishustinii (mi.shu.sti'ni.i. N.L. masc. gen. n. *mishustinii* of Mishustin, named in honour of E. N. Mishustin, a noted Russian soil microbiologist).

Basonym: *Prosthecomicrobium mishustinii* Vasil'eva *et al.* 2009.

Characteristics are as described by Vasil'eva *et al.* (1991). Cells are thick, short rods, 0.6–1.2 µm in diameter and

0.8–1.5 µm in length. Cells are unicellular or paired. Non-motile. Prosthecae are from 0.2 to 0.65 µm in length. Cells reproduce by budding. Cells are aerobic and catalase- and oxidase-positive. Uses many mono- and disaccharides and sugar alcohols, some organic acids as well as amino acids as energy sources for growth. Yeast extract or B-vitamins are required for growth. Optimum growth temperature range is 28 to 30 °C at neutral pH. Colonies are yellowish-orange, have an entire margin and a thick consistency so that they can be removed from agar medium. Colony variants lacking pigment may be produced. Widespread in soil, manure and other organically enriched habitats. The DNA G + C content of the type strain is 63.7–65.2 mol% (*T_m*).

The type strain is strain 17^T (=VKM B-2499^T =CCM 7569^T).

Description of *Bauldia* gen. nov.

Bauldia (Baul'di.a. N.L. fem. n. *Bauldia* of Bauld, named in honour of John Bauld, an Australian microbiologist who isolated, investigated and named members of the genus *Prosthecomicrobium* and *Planctomyces maris*).

Gram-negative, budding prosthecate bacteria with numerous short appendages, less than 0.65 µm in length, extending from the cell surface. Aerobic and heterotrophic. Various carbon sources can be used for growth including

some mono- and disaccharides and organic acids. Found in brackish water and soil habitats. DNA G+C content (mol%) is 66 to 69. The type species is *Bauldia litoralis*.

Description of *Bauldia litoralis* comb. nov.

Bauldia litoralis (li.to.ra'lis. L. fem. adj. *litoralis* living in water, aquatic).

Basonym: *Prosthecomicrobium litorale* Bauld *et al.* 1983.

Characteristics are as described by Bauld *et al.* (1983). The DNA G+C content of the type strain is 66–67 mol% (buoyant density).

The type strain is strain 524-16^T (=NCIB 2233^T =ATCC 35022^T).

Description of *Bauldia consociata* comb. nov.

Bauldia consociata (con.so.ci.a'ta. L. part. fem. adj. *consociata* associated, intended to mean living in a community).

Basonym: *Prosthecomicrobium consociatum* Vasil'eva *et al.* 2009.

Characteristics are as described by Vasil'eva *et al.* (1991). Cells are short, thick rods, 0.5–1.0 µm in diameter and 0.8–1.35 µm in length. Prosthecae are 0.2 to 0.25 µm in length and arranged in five rows along cells. End view of cells shows a five-pointed star. Non-motile. Reproduce by budding. Aerobic and catalase- and oxidase-positive. Chemoheterotrophic. Uses some monosaccharides as carbon and energy sources for growth. Does not hydrolyse cellulose or other polysaccharides. Some sugar alcohols, organic acids of the TCA cycle and methanol and methylamine can be used as carbon sources. Yeast extract or B-vitamins are required for growth. Optimum growth temperature is 28–30 °C at neutral pH. Colonies are small (up to 1 mm in diameter) and grey with an entire margin. Widespread in soil habitats and compost where cellulolytic activity is high. The DNA G+C content of the type strain is 66.0–68.5 mol% (*T_m*).

The type strain is strain 11^T (=VKM B-2498^T =CCM 7594^T).

Acknowledgements

The authors wish to thank J. P. Euzéby for his advice on the proper nomenclature and A. Ventosa and H. Christensen for their editorial assistance. In addition, the authors are grateful to Dow Agrosiences for partial support to enable B.Y. to conduct this research. B.Y. is participating in a University of Washington–University of Queensland graduate student exchange program. G.E.O. was supported in part by the University of Washington Developmental Biology Training Grant National Institutes of Health Training Grant T32HD007183-25.

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