'*Candidatus Aquirestis calciphila*' and '*Candidatus Haliscomenobacter calcifugiens*', filamentous, planktonic bacteria inhabiting natural lakes

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Filamentous bacteria frequently occurring in the pelagic zone of natural freshwater lakes and ponds were previously identified as being related to *Haliscomenobacter hydrossis* based upon their 16S rRNA gene sequences. These bacteria exhibit a specific morphology characterized by the formation of straight, stick-like filaments of variable length (5 to > 100 µm) and quite stable, but narrow, width (0.25 to 0.35 µm). Bacteria with these morphological characteristics form a monophyletic but broad phylogenetic group with a maximal divergence of 16S rRNA gene sequences of 12.0%. This monophyletic group consists of at least three monophyletic subclusters. *H. hydrossis* is affiliated to one of these subclusters and represents the sole recognized species affiliated to the broad monophyletic group. '*Candidatus Haliscomenobacter calcifugiens*' and '*Candidatus Aquirestis calciphila*' are uncultured representatives of the other two subclusters and have 16S rRNA gene sequence dissimilarities of 5.4% and 8.2%, respectively, with the type strain of *H. hydrossis*. '*Candidatus H. calcifugiens*' and '*Candidatus A. calciphila*' have a 16S rRNA gene sequence dissimilarity of 8.5%. These large ribosomal divergences justify the classification of these environmentally important bacteria as a novel species and a new genus, respectively. Intensive attempts to cultivate these filamentous bacteria have resulted in the establishment of mixed cultures, however, attempts to establish pure cultures have failed.

The bacterioplankton of freshwater habitats is mainly comprised by bacterial groups that do not contain validly described species or cultivated representatives (Zwart *et al.*, 2002). One of these groups is the so-called LD2 group which was described in 2002 based on four environmental 16S rRNA gene sequences obtained from two Dutch lakes (Zwart *et al.*, 2002). Phylogenetic analysis of these environmental sequences indicated that the LD2 bacteria were related to *Saprospira grandis* and *Haliscomenobacter hydrossis* of the phylum *Bacteroidetes* (Zwart *et al.*, 2002). Pernthaler *et al.* (2004) demonstrated by fluorescent *in situ* hybridization (FISH) of samples obtained from a single lake that bacteria affiliated to the LD2 group share a characteristic filamentous morphology with *H. hydrossis*. We attempted to isolate bacteria possessing this *H. hydrossis*-like morphology by utilizing various cultivation

methods and media. This resulted in the enrichment of the targeted filamentous bacteria and the establishment of mixed cultures in which up to 42% of the total number of bacterial cells were of the targeted bacterial morphotype. However, the establishment of pure cultures of the targeted bacteria failed despite the achievement of enrichments of at least one order of magnitude (Schauer & Hahn, 2005).

In order to obtain insights into the phylogeny of the filamentous bacteria characterized by an *H. hydrossis*-like morphology, primers for the specific amplification of the 16S rRNA gene sequences of the targeted bacteria were developed by a step-wise approach (Schauer & Hahn, 2005). By using these primers, a large number of sequences potentially representing the targeted filamentous bacteria were obtained from enrichment cultures and from various samples from freshwater habitats. Based on these sequences, a suite of five nested FISH probes was developed (Table 1) and these probes were used for verification that the obtained sequences originated from the targeted bacteria with the *H. hydrossis*-like morphology. By this approach, it was demonstrated that a broad phylogenetic group, provisionally designated the SOL cluster, exclusively harbours bacteria possessing the typical *H. hydrossis*-like morphology (Schauer & Hahn, 2005).

Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; FISH, fluorescent *in situ* hybridization.

The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequences of '*Candidatus Aquirestis calciphila*' and '*Candidatus Haliscomenobacter calcifugiens*' are AJ786341 and AJ786327, respectively.

Table 1. FISH probes targeting the 16S rRNA gene for detection and discrimination of *H. hydrossis*, 'Candidatus *H. calcifugiens*' and 'Candidatus *A. calciphila*' GKS2-217, LD2 and HAL are subclusters of the SOL cluster (Schauer & Hahn, 2005). *H. hydrossis* is affiliated with the HAL subcluster. +, Positive; –, negative.

Probe	Sequence	Hybridization with			Specificity	Reference
		<i>H. hydrossis</i> DSM 1100 ^T	'Candidatus <i>H. calcifugiens</i> '	'Candidatus <i>A. calciphila</i> '		
GKS-847	5'-TTCGCTTGG ACACACAATC-3'	–	+	–	GKS2-217 subcluster	Schauer & Hahn (2005)
LD2-1261	5'-GGCTCCGCTTCACAGCTT-3'	–	–	+	LD2 subcluster	Schauer & Hahn (2005)
LD2-739	5'-GCGTCAATACAGATCCAG-3'	–	–	+	LD2 subcluster	Pernthaler <i>et al.</i> (2004)
HAL-844	5'-CGCTTGGACACTCACTCC-3'	+	–	–	HAL subcluster	Schauer & Hahn (2005)
HHY-441	5'-CCAGATTCTTCCCAAGC-3'	+	–	–	<i>H. hydrossis</i>	Schauer & Hahn (2005)
HHY-655	5'-GCCTACCTCAACCTGATT-3'	+	–	–	<i>H. hydrossis</i> , (HAL)*	Wagner <i>et al.</i> (1994)
SOL-852	5'-ACGCTTTCGCTTGGACAC-3'	+	+	+	SOL cluster†	Schauer & Hahn (2005)

*The probe matches with *H. hydrossis* and some other sequences within the HAL subcluster.

†The probe has unspecific matches with strains outside of the SOL cluster (Schauer & Hahn, 2005). The matching organisms outside the SOL cluster differ in morphology from the SOL bacteria.

Phylogeny of bacteria with *H. hydrossis*-like morphology

Sequences within the so-called SOL cluster are affiliated with the phylum *Bacteroidetes*. The SOL cluster is characterized by species with a minimum 16S rRNA gene sequence similarity of 88 % and consists of at least three monophyletic subclusters, provisionally designated the LD2, HAL and GKS2-217 subclusters (Schauer & Hahn, 2005). The HAL subcluster is the only subcluster that contains a recognized species, *H. hydrossis* (van Veen *et al.*, 1973). The HAL subcluster is characterized by a minimum 16S rRNA gene sequence similarity of 98.7 %, suggesting that all members of this subcluster belong to the species *H. hydrossis* (Rossello-Mora & Amann, 2001). Members of the other two subclusters are more distantly related to *H. hydrossis*. Sequences affiliated with the GKS2-217 subcluster share 16S rRNA gene sequence similarities of 93.8–95.5 % with members of the HAL subcluster. 16S rRNA gene sequences affiliated with the LD2 subcluster have sequence similarities of 90.1–92.7 % with those of the HAL subcluster. The minimal 16S rRNA gene sequence similarities within the GKS2-217 and LD2 subclusters are 97.6 % and 99.7 %, respectively. The low between-subcluster similarity values indicate that members of the different subclusters are not representatives of *H. hydrossis* and thus justify the proposal to classify one member of the GKS2-217 subcluster (reference clone MS-oKlaff1-G) as a candidate for a novel *Haliscomenobacter* species and propose the designation 'Candidatus *Haliscomenobacter calcifugiens*'. Furthermore, we propose to classify one member of the LD2 subcluster (reference clone MS-Falk1-L) as a candidate for a novel species in a new genus and propose the designation 'Candidatus *Aquirestis calciphila*'. The novel taxa 'Candidatus *H. calcifugiens*' and 'Candidatus *A. calciphila*' share 91.5 % 16S rRNA gene sequence similarity with each other and 94.6 and 91.8 %, respectively, with the type strain of *H. hydrossis*. Phylogenetic analysis of 16S rRNA gene sequences (Fig. 1) revealed that all three taxa cluster together within the family 'Saprospiraceae' (Garrity *et al.*, 2004).

Morphological traits

The application of FISH probes specific for the entire SOL cluster and its three subclusters, respectively, to samples from the pelagic zones of 115 freshwater habitats located on four continents and in three climatic zones revealed that all detected members of the SOL cluster share the same distinct morphology (Fig. 2). All these bacteria appeared as straight, stick-like filaments of variable length ranging from 5 to > 100 µm and of quite stable, but narrow, width of 0.25–0.35 µm. A sheath enclosing the filaments was sometimes visible in 4',6'-diamidino-2-phenylindole (DAPI)-stained preparations (epifluorescence microscopy). Branching filaments were never detected with the probes specific for the SOL cluster or its subclusters.

Fig. 1. Neighbour-joining tree based on almost full-length 16S rRNA gene sequences of species affiliated with the phylum Bacteroidetes. Bootstrap values represent percentage support of the nodes based on 1000 resamplings (only values > 60 are shown). Three sequences of *Actinobacteria* (GenBank accession numbers X77435, U09761 and U09763) served as an outgroup (not shown). Note that *Haliscomenobacter hydrossis*, '*Candidatus H. calcifugiens*' and '*Candidatus A. calciphila*' are representatives of the so-called HAL, GKS2-217 and LD2 subclusters, respectively, which together constitute the so-called SOL cluster (Schauer & Hahn, 2005). Bar, 10% estimated sequence divergence.

We purchased the type strain of *H. hydrossis* (DSM 1100^T) for comparison of morphological traits but found that this strain had lost its ability to form a sheath (van Veen *et al.*, 1973). It seemed that the loss of this trait resulted in the formation of erratically curved filaments which lacked the morphological characteristics originally described for this strain (van Veen *et al.*, 1973). The filamentous bacteria detected by the specific FISH probes in the investigated

freshwater lakes shared several of the morphological characteristics originally described for the *H. hydrossis* type strain (van Veen *et al.*, 1973; Eikelboom, 1975), but showed smaller filament width than that described for the type strain (van Veen *et al.*, 1973). These differences in the results of filament width measurements could result from the application of different sizing methods. Furthermore, we searched for *H. hydrossis* filaments in several samples from natural freshwater habitats with a species-specific probe (Schauer & Hahn, 2005) but never detected any probe-positive cells.

Fig. 2. Photomicrograph of a DAPI-stained water sample from the oligotrophic Lake Attersee (Austria). The image shows a 46 µm long filament of '*Candidatus A. calciphila*' surrounded by several much smaller cells of planktonic bacteria.

A morphological comparison of filaments detected by the different subcluster-specific probes did not reveal any discriminatory features. Thus, bacteria affiliated with the SOL cluster (including the originally described *H. hydrossis* phenotype) can be discriminated from other bacteria by their unique morphology, but members of the three different subclusters cannot be discriminated from each other on the basis of morphological traits.

Physiology

Little is known about the physiology of these bacteria due to the lack of pure cultures. In order to estimate the growth rate of '*Candidatus A. calciphila*' under natural conditions, 0.2 µm-filtered lake water was inoculated with predator-free (without water fleas and other metazooplankton species) water from Lake Mondsee, Austria, and incubated at 20 °C. The growth of the targeted bacteria was monitored by microscopic observations. '*Candidatus A. calciphila*' bacteria grew in the filtered lake water with doubling times of 3.0 days (growth rate 0.23 day⁻¹) which indicates that the *in situ* growth rates of these bacteria are low. Growth under anoxic conditions was not observed, while control treatments

incubated under oxic conditions showed a clear increase in cell numbers. These observations may indicate an obligately aerobic physiology for 'Candidatus A. calciphila'.

Ecology

A large number of freshwater habitats have been investigated for the presence of the three SOL subclusters by specific FISH probes (Schauer *et al.*, 2005). Bacteria affiliated to the SOL subcluster were detected in 73 % of the 115 investigated habitats. The LD2 subcluster, which is represented by 'Candidatus A. calciphila', was detected in 62 % of all investigated samples, while the GKS2-217 subcluster, represented by 'Candidatus H. calcifugiens', was detected in 12 % of the investigated samples. The HAL subcluster, represented by *H. hydrossis*, was detected in 22 % of the hybridized samples. Members of the subclusters represented by 'Candidatus A. calciphila' and 'Candidatus H. calcifugiens' were never observed to co-occur in the same habitat, while members of the HAL subcluster, represented by *H. hydrossis*, usually co-occurred with members of one of the other two subclusters. Multivariate statistical analyses revealed that water chemistry parameters mainly control the occurrence of 'Candidatus A. calciphila' and 'Candidatus H. calcifugiens' in stagnant freshwater habitats. 'Candidatus A. calciphila' (i.e., subcluster LD2) is restricted to hard-water habitats characterized by medium to high concentrations of calcium and magnesium carbonate, with conductivity values $>60 \mu\text{S cm}^{-1}$ and pH values ≥ 7.7 . 'Candidatus A. calciphila' was also observed in the polysaline Lake Qinghai, China (salinity 20 g l^{-1} ; Wu *et al.*, 2006), which is the seventh largest saline lake in the world. Thus, this taxon is not restricted to freshwater habitats (salinity $<1 \text{ g l}^{-1}$). In contrast, 'Candidatus H. calcifugiens' is restricted to soft-water (low concentrations of calcium and magnesium carbonate) habitats and was never detected in saline lakes. The stagnant systems inhabited by 'Candidatus H. calcifugiens' are characterized by low concentrations of calcium and magnesium carbonate with conductivity values $\leq 60 \mu\text{S cm}^{-1}$ and pH values in the range 6.4–7.3. Members of both groups (as well as members of the HAL subcluster represented by *H. hydrossis*) were never observed in acidic freshwater habitats with pH values <6 .

'Candidatus H. calcifugiens' is characterized by a narrow ecological amplitude. The soft-water habitats preferred by this taxon have been found in the Austrian Alps and in northern Sweden (Schauer *et al.*, 2005). In contrast, 'Candidatus A. calciphila' was found over a broad range of habitat types which indicates a wide ecological amplitude. For instance, this taxon was detected in a hypertrophic part of Lake Taihu located in the subtropical part of China, in Lake Victoria and Lake Tanganyika located in tropical Africa and in the oligotrophic Lake Attersee in Austria (Fig. 2).

Both *Candidatus* organisms seem to be restricted to the pelagic zone of stagnant inland waters and were never reported from soil or marine habitats. Both organisms were detected over the entire water column of Lake Mondsee,

which has a maximum depth of 68 m. Furthermore, 'Candidatus A. calciphila' was detected year-round in the same lake, but showed pronounced seasonal differences in abundance. In two consecutive years differing strongly in climatic conditions, a very similar seasonal pattern of population dynamics was observed. Each year, the 'Candidatus A. calciphila' population formed a strong spring peak and showed minor population peaks in summer and early autumn. By contrast, the HAL group (to which *H. hydrossis* is affiliated) was detected in the same lake only during a short period of a few weeks in early autumn.

'Candidatus A. calciphila' was also detected in low numbers in running water systems fed with water from lakes inhabited by populations of these bacteria. On the other hand, these bacteria were not detected in running waters that lacked lakes or ponds located upstream. This observation seems to indicate that the primary habitat of these bacteria is the pelagic zone of stagnant inland waters.

Both 'Candidatus A. calciphila' and 'Candidatus H. calcifugiens' represent an important part of the freshwater bacterioplankton and appear in the water column of freshwater habitats with relative abundances of $<1\%$ to at least 11 % of total bacterial numbers (Schauer & Hahn, 2005). The observed total cell numbers for these taxa ranged from several hundreds to $2 \times 10^5 \text{ cells ml}^{-1}$. Due to their filamentous morphologies and typical mean filament lengths of 20–50 μm , these bacteria contribute over proportionally to the total bacterial biomass in freshwater habitats. These bacteria comprise about 40 % of the total bacterial biovolume in some habitats (Schauer & Hahn, 2005).

Recently, water flea (*Daphnia* spp.) and other metazooplankton species were identified as important predators of a 'Candidatus A. calciphila' population in the oligo-mesotrophic Lake Mondsee (Schauer *et al.*, 2006). On the other hand, these bacteria were found to be resistant to predation by bacterivorous protists (flagellates and ciliates) which are usually the major predators of planktonic bacteria in freshwater systems (Hahn & Höfle, 2001).

Biogeography

'Candidatus A. calciphila' is a cosmopolitan inhabitant of hard-water lakes. This taxon was detected in habitats located in Oceania (Australia, New Zealand), Central America (Mexico), Africa (Lake Victoria, Lake Tanganyika), Eurasia (Austria, China), as well as in habitats located in the temperate, subtropical and tropical zones. Furthermore, this taxon was also detected in a high mountain lake located at an altitude of 4987 m on the Tibetan Plateau (Schauer *et al.*, 2005; Wu *et al.*, 2006). Altogether, these observations indicate that 'Candidatus A. calciphila' may inhabit all appropriate hard-water lakes located on all continents and in all climatic zones.

'Candidatus H. calcifugiens' has so far been exclusively detected in soft-water lakes located in Austria and Sweden.

However, soft-water lakes suitable for this taxon located outside of these two regions have not so far been investigated for the presence of '*Candidatus* H. calcifugiens'. Thus, a wider biogeographic distribution for this taxon cannot be ruled out.

Cultivation

Despite extensive efforts to grow representatives of subclusters LD2 and GKS2-217 in pure culture by using various media and isolation methods (Schauer & Hahn, 2005), pure cultures have not yet been successfully established. However, until cultivation of these important representatives of freshwater bacterioplankton is achieved or additional phenotypic data become available, we assign members of the two species-like subgroups of the SOL cluster to the provisional *Candidatus* status as proposed by Murray & Stackebrandt (1995).

Description of '*Candidatus* Aquirestis calciphila'

'*Candidatus* Aquirestis calciphila' [A.qui.res'tis. L. n. *aqua* water; L. fem. n. *restis* thread, rope; N.L. fem. n. *Aquirestis* water thread; cal.ci'phi.la. L. fem. n. *calx* limestone (calcium carbonate); Gr. adj. *philos* loving, friendly to; N.L. fem. adj. *calciphila* loving limestone].

[(*Bacteroidetes*) NC; F; NAS (GenBank number AJ786341), oligonucleotide sequence complementary to unique region of 16S rRNA 5'-AAGCTGTGAAGCGGAGCC-3'; FL (freshwater and saline lakes, planktonic); Aer, long straight filaments with width ≤ 0.35 μ m; M]. Schauer & Hahn, *Appl Environ Microbiol* 71:1931–1940, 2005.

Description of '*Candidatus* Haliscomenobacter calcifugiens'

'*Candidatus* Haliscomenobacter calcifugiens' [cal.ci.fu'-gi.ens. L. fem. n. *calx* limestone (calcium carbonate); L. part. pres. *fugiens* fleeing, shy; N.L. part. pres. *calcifugiens* fleeing from limestone].

[(*Bacteroidetes*, genus *Haliscomenobacter*); NC; F; NAS (GenBank AJ786327), oligonucleotide sequence complementary to unique region of 16S rRNA 5'-CGGATTGTGTGTCCAAGCGAA-3'; FL (freshwater lakes with soft-water, planktonic); Aer, long straight filaments with width ≤ 0.35 μ m; M]. Schauer & Hahn, *Appl Environ Microbiol* 71:1931–1940, 2005.

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