'*Candidatus* Planktophila limnetica', an actinobacterium representing one of the most numerically important taxa in freshwater bacterioplankton

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Actinobacteria often constitute a large fraction of the bacterioplankton in freshwater systems. Cultivation-independent methods have revealed that the so-called *acl* lineage frequently represents the most numerous taxon among assemblages of freshwater *Actinobacteria* and even among total freshwater bacterioplankton. Bacteria affiliated with this uncultivated lineage have been detected in freshwater habitats located in various continents and climatic zones but have never been found among terrestrial or offshore marine systems. So far, this ecologically important lineage of freshwater *Actinobacteria* is not represented by a recognized taxon. In this study, we established a stable mixed culture containing a strain affiliated with the *acl* lineage from a freshwater lake in Austria. The proportion of the strain in the culture could be increased by manipulation of the medium composition by more than one order of magnitude, however all subsequent attempts to isolate this strain into pure culture were unsuccessful. Some of the phenotypic traits of this *acl* strain were determined and its taxonomic position within the *Actinobacteria* was analysed. Phylogenetic analysis of this organism's 16S rRNA gene revealed a distant relationship with cultivated organisms and recognized species (89 % gene sequence similarity with the latter). Furthermore, this analysis did not support a clear assignment of the strain to any of the recognized families within the phylum *Actinobacteria*. It is suggested that a candidate taxon, '*Candidatus* Planktophila limnetica' is established to represent this strain.

As late as the mid 1990s, members of the phylum *Actinobacteria* were not regarded as typical inhabitants of freshwaters (Rheinheimer, 1980). This knowledge was based entirely on cultivation experiments, which indicated low numbers of Gram-positive bacteria in lakes and ponds. Gram-positive strains isolated from freshwater plankton

were also common in terrestrial habitats, thus suggesting their allochthonous origin (Rheinheimer, 1980). Cultivation-independent methods radically changed our view regarding the significance of *Actinobacteria* in freshwater systems (Glöckner *et al.*, 2000; Zwart *et al.*, 2002). Such methods found that bacteria from this phylum frequently constitute a large fraction of freshwater bacterioplankton, accounting for 10–60 % of the total DAPI-stained prokaryotes (Glöckner *et al.*, 2000; Sekar *et al.*, 2003; Warnecke *et al.*, 2004; Allgaier & Grossart, 2006). Several actinobacterial lineages have been revealed to be common in freshwater systems, as defined by analysis of 16S rRNA gene sequences of uncultured *Actinobacteria*, amplified with the PCR from environmental DNA samples (Zwart *et al.*, 2002; Warnecke *et al.*, 2004; Allgaier & Grossart, 2006; Wu *et al.*, 2007). Most of these taxa are exclusive to freshwater habitats, indicating an indigenous

Abbreviations: CARD-FISH, catalysed reporter deposition fluorescence *in situ* hybridization; DAPI, 4',6-diamidino-2-phenylindol dihydrochloride; FISH, fluorescent *in situ* hybridization; NJ, neighbour-joining; ML, maximum-likelihood.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of '*Candidatus* Planktophila limnetica' strain MWH-EgelM2-3.acl is FJ428831.

Three supplementary figures, a supplementary table and details of methods used to isolate strain MWH-EgelM2-3.acl are available with the online version of this paper.

occurrence in freshwater systems (Zwart *et al.*, 2002). The enormous ecological significance of planktonic *Actinobacteria* in freshwater systems stands in strong contrast to the slow development of the taxonomic knowledge of this bacterial group. Strains representing indigenous freshwater *Actinobacteria* have only recently been cultivated (Hahn *et al.*, 2003) and their taxonomic description (Hahn, 2009) only accounts for a small fraction of the diversity represented by freshwater lineages within this phylum.

Amongst the groups of freshwater *Actinobacteria* characterized by cultivation-independent methods, organisms of the acI lineage (Warnecke *et al.*, 2004) often represent the most numerous taxa (Zwart *et al.*, 2002; Warnecke *et al.*, 2005; Newton *et al.*, 2007). The acI group was originally described as the ACK-M1 lineage by Zwart *et al.* in 2002, and represents a phylogenetically broad group of organisms displaying $\geq 93\%$ sequence similarity at the 16S rRNA gene sequence level. This lineage is globally distributed and occurs in a variety of freshwater systems that differ by physical, chemical and biological parameters (Lindström *et al.*, 2005; Newton *et al.*, 2007).

Here, we describe the establishment of a stable mixed culture containing a strain affiliated with the acI lineage. All efforts to isolate and cultivate this strain as a pure culture have so far been unsuccessful. Due to the lack of a pure culture, the establishment of the candidate taxon '*Candidatus Planktophila limnetica*' is proposed according to Murray & Stackebrandt (1995).

Cultivation

A cultivation experiment inoculated with water from Lake Egelsee (small freshwater lake, pH 7.6, conductivity $376 \mu\text{S cm}^{-1}$, moderate humic content, located near Salzburg, Austria) resulted in the establishment of a mixed bacterial culture, designated MWH-EgelM2-3. This culture was obtained by using NSY medium and the filtration acclimatization method (Hahn *et al.*, 2004). A detailed investigation of the composition of this culture using fluorescence *in situ* hybridization (FISH) and PCR for different phylogenetic markers (see Supplementary Material and Supplementary Fig. S1 in IJSEM Online for details) revealed the presence of a strain (designated MWH-EgelM2-3.acI) affiliated with the acI lineage (Warnecke *et al.*, 2004), which we describe here as '*Ca. Planktophila limnetica*'. Besides the acI strain, the PCR and FISH analyses identified another actinobacterial strain ('*Candidatus Limnoluna rubra*', Hahn, 2009), and a *Polynucleobacter necessarius* subsp. *asymbioticus* strain as present in this mixed culture. All attempts to isolate the '*Ca. Planktophila limnetica*' strain in pure culture were unsuccessful. Detailed descriptions of the establishment of the mixed culture, analysis of its population and the attempts to establish a pure culture are presented in the Supplementary Material (available in IJSEM Online).

Investigations by FISH with the acI-specific probe acI-852 and two helper oligonucleotides (Warnecke *et al.*, 2005)

revealed that '*Ca. Planktophila limnetica*' only contributed $<0.1\%$ to the total cell numbers of culture MWH-EgelM2-3 when cultivated in NSY medium (Hahn *et al.*, 2004). In order to test whether the proportion of the acI bacteria in the mixed culture could be increased, we tested the effects of NSY medium supplemented with 20 separate substrates (Table 1). We found several substrates that increased the proportion of the acI cells by greater than one order of magnitude. The addition of L-alanine was by far the most effective substrate tested, increasing the proportion of acI cells to 5.6% of total cells.

Phylogeny

Using the 16S rRNA gene sequence of '*Ca. Planktophila limnetica*' as a BLAST search query, it was found that this organism appeared to be only distantly related to any recognized bacterial species. The search revealed that the 763 most closely related sequences represented uncultured *Actinobacteria* (analysis performed on 23 September 2008)

Table 1. Taxon-specific composition of culture MWH-EgelM2-3 grown in liquid NSY medium (0.7 g l^{-1}) supplemented with 0.5 g l^{-1} of different substrates

The composition was determined by CARD-FISH with probes acI-852 (specific for the acI group; Warnecke *et al.*, 2005) and HGC69a (specific for *Actinobacteria*; Roller *et al.*, 1994). The non-actinobacterial proportion was comprised by a *Polynucleobacter* strain. A comparison of absolute cell numbers was avoided due to expected differences in the growth efficiency on the various substrates.

Substrate	<i>Actinobacteria</i> *	acI*	acI†
Acetate	65.7	0.8	1.2
L-Alanine	87.3	5.6	6.4
L-Aspartate	91.8	1.8	2.0
Betaine	73.5	1.5	2.0
L-Fucose	86.1	0.7	0.8
Fumarate	83.4	0.6	0.8
D-Galacturonic acid	77.3	1.5	2.0
D-Glucose	88.4	<0.1	NA
D-Glycerate	49.8	2.6	5.2
Glycolate	63.6	2.0	3.2
Glyoxylate	62.0	0.3	0.5
Humic acids (Sigma)	59.5	1.8	2.9
L-Isoleucine	87.9	1.4	1.6
Isovalerate	94.2	3.1	3.2
D-Lyxose	87.7	2.1	2.4
Malate	72.2	1.0	1.4
Oxalate	75.2	2.8	3.7
Pyruvate	58.2	0.7	1.2
L-Serine	72.3	2.3	3.1
Succinate	62.3	2.8	4.5

*Expressed as a percentage of total cell numbers.

†Expressed as a percentage of *Actinobacteria*.

and the similarity values obtained for these sequences ranged from 100 % (no mismatch) to 89 % sequence similarity. The closest 16S rRNA gene sequence similarity value between '*Candidatus* Planktophila limnetica' and a cultured organism was that between the novel taxon and *Sporichthya polymorpha* IFO 12702^T (89 % sequence similarity).

To reveal the phylogenetic position of '*Ca.* Planktophila limnetica', we performed an analysis of 73 sequences representing all of the recognized families currently found within the phylum *Actinobacteria*. The reference sequences were aligned in ARB (Ludwig *et al.*, 2004) and exported to other programs for tree construction. Neighbour-joining (NJ) trees were calculated with MEGA4 (Tamura *et al.*, 2007) and maximum-likelihood (ML) trees were obtained using the RAxML web-server (Stamatakis *et al.*, 2008). Phylogenies of the 16S rRNA gene reconstructed with both algorithms demonstrated the affiliation of the strain with the phylum *Actinobacteria* and the order *Actinomycetales*. The NJ tree (Fig. 1) and the ML tree (see Supplementary Fig. S2 in IJSEM Online) constructed with the same representatives of the order *Actinomycetales*, resulted in different tree topologies with neither topology supporting the affiliation of the acI strain to a specific suborder. In the ML tree, the 16S rRNA gene sequence of '*Ca.* Planktophila limnetica' formed a well-supported clade with *Sporichthya polymorpha* IFO 12702^T (90 % bootstrap value), however this organism belongs to a suborder (*Frankineae*) that does not appear as a monophyletic group in the tree. The results of the phylogenetic analyses must be interpreted with caution as the set of 16S rRNA gene sequences representing the type species for each family contained many old reference sequences of relatively low sequence quality. For example, the sequences used for the construction of these 16S rRNA gene sequence phylogenies (Fig. 1 and Supplementary Fig. S2) contained 163 'N' in total and many unusual alignment gaps, which could result from missing nucleotides. Reference sequences of higher quality, as well as those from organisms more closely related to the *Candidatus* taxon will be required in order to conduct a better analysis of the phylogenetic position of this new taxon.

On the other hand, both trees demonstrated the affiliation of '*Ca.* Planktophila limnetica' to the 'acI lineage' (or 'acI clade'), a taxon established for environmental 16S rRNA gene sequences (Warnecke *et al.*, 2004). The acI lineage represents a phylogenetically broad cluster, exhibiting ≥ 93 % 16S rRNA gene sequence similarity. This cluster is currently subdivided into three genus-like subgroups (acI-A, acI-B and acI-C), each of which is characterized by internal 16S rRNA gene sequence similarity values of ≥ 95 % (Newton *et al.*, 2007). Recently, groups acI-A and acI-B were subdivided further into more narrow clusters (Newton *et al.*, 2007), resulting in 11 clades (AI to BIV) each characterized by >97.9 % within-clade 16S rRNA gene sequence similarity (see Supplementary Fig. S3 and the Supplementary Material).

The 16S rRNA gene sequence of '*Ca.* Planktophila limnetica' was identical to a sequence (clone MIIF1, GenBank accession number EU117876) affiliated with clade acI-AII (Newton *et al.*, 2007). This clade represents a species-like taxon characterized by a minimum 16S rRNA gene sequence similarity of 99.4 % (see Supplementary Fig. S3). All of the 16S rRNA gene sequences affiliated with the acI-AII group share the oligonucleotide sequence 5'-GAAACTTGGTGGCATCRCCG-3' (positions 183 to 193, *Escherichia coli* numbering, Supplementary Table S1 in IJSEM Online).

Phenotypic traits

'*Ca.* Planktophila limnetica' possessed cells with diameters of 0.4–0.5 μm and mean lengths of 1.2 μm , with a slightly C-shaped morphology (Fig. 2). The mixed culture containing '*Ca.* Planktophila limnetica' was red pigmented, however, it is not known if this strain contributes to this pigmentation or if it is caused by the second actinobacterial strain present in the culture. Due to the lack of a pure culture, assimilation tests as well as other standard investigations for the characterization of physiological traits could not be performed. The observed growth responses in NSY media supplemented with 20 separate substrates (Table 1) must also be interpreted with caution. The recorded responses of '*Ca.* Planktophila limnetica' may have resulted from a complex interplay of the three strains present in the mixed MWH-EgelM2-3 culture. In some cases, the increased relative abundance of '*Ca.* Planktophila limnetica' appeared to be favoured by weaker growth of the second actinobacterial strain (e.g. supplementation with D-glycerate, glycolate and humic acids). In cases where this growth shift did not occur, the assimilation of substrate by '*Ca.* Planktophila limnetica' might have been taking place and can be assumed (e.g. for L-alanine, isovalerate and D-lyxose).

Ecology

Members of the acI clade have been observed in the water columns of a broad variety of freshwater habitats (Warnecke *et al.*, 2004, 2005; Lindström *et al.*, 2005; Allgaier & Grossart, 2006; Newton *et al.*, 2007), ranging from acidic to alkaline systems and even including lakes located at altitudes of >4400 m above sea level (Wu *et al.*, 2006). In an ecological study of several phylogenetic groups of bacteria present in 16 lakes along a salinity gradient from freshwater to hypersaline, the distribution of a major subgroup of the acI lineage (ACK-M1) was restricted to freshwater and weakly oligosaline lakes (Wu *et al.*, 2006). It has been demonstrated by Newton *et al.* (2007) that members of the acI-AII clade, to which '*Ca.* Planktophila limnetica' belongs, exhibit a preference for more alkaline lakes. During a study of 18 lakes representing a pH gradient from 5.1 to 8.9, Newton *et al.* (2007) found that the distribution of the acI-AII clade was restricted to nine of them, which ranged in pH from 6.5 to 8.9. 16S rRNA

Fig. 1. Neighbour-joining tree calculated with 16S rRNA gene sequences (1311 aligned sequence positions, ranging from 175 to 1443, *E. coli* numbering) illustrating the phylogenetic position of the cultured acl strain 'Ca. Planktophila limnetica'. Almost every family of the order Actinomycetales is represented by the type strain for the type species of the respective family. The family Frankiaceae is not represented because no type strain was designated for the type species of the family. The tree was calculated with 1000 bootstrap iterations and *Patulibacter* (*Rubrobacterales*) served as an outgroup. Note that the suborders Frankineae and Actinomycineae do not appear as monophyletic groups in the tree, therefore labelling of these taxa in the tree was omitted. Interestingly, these suborders also did not appear as monophyletic groups in previously published phylogenetic trees (Busti *et al.*, 2006; Cavaletti *et al.*, 2006). Exclusion of the three environmental sequences (acl group) from the tree calculation did not change the overall tree topology.

Fig. 2. Epifluorescence microscope image of cells of 'Ca. Planktophilia limnetica' in the MWH-EgelM2-3 culture hybridized with the acI-specific CARD-FISH probe acI-852, and counter stained with the nucleic acid dye DAPI. The image represents an overlay of two images obtained from excitation of DAPI (blue signals) and FITC (label resulting from the probe binding). The probe-positive cells, i.e. 'Ca. Planktophilia limnetica', appear with a pink colour.

clones of the acI-AII clade were most abundant in lakes with pH values of 8.6, 8.8 and 8.9, where such sequences contributed 18–22 % of the total acI clones present in each of these three samples. The pH values measured in Lake Egelsee (7.1–8.0; mean 7.7; 6 samplings), from which culture MWH-EgelM2-3 was obtained, coincided with the pH preference demonstrated for the acI-AII clade.

The acI-A lineage was detected in ten oligo- to mesotrophic mountain lakes located between 913 and 2799 m above sea level during a study by Warnecke *et al.* (2005). This study employed methods that were unable to capture the fine-scale phylogenetic resolution of the acI-A group, but the highest relative abundance of acI-A cell types was found in a lake of pH 7.3 located at an altitude of 2344 m where they represented ~25 % of total bacterial numbers. Altogether these observations indicate that 'Ca. Planktophilia limnetica' is a representative of planktonic bacteria dwelling in freshwater and possibly slightly oligosaline inland waters (see Supplementary Table S1) and possessing a preference for neutral to alkaline pH values.

Description of 'Candidatus Planktophilia limnetica'

'Candidatus Planktophilia limnetica' [Plank.to'phi.la. N.L. neut. n. *plankton* plankton; N.L. fem. adj. *phila* friendly to; N.L. fem. n. *Planktophilia* the friend (fem.) of plankton; lim.ne'ti.ca. N.L. fem. adj. *limnetica* pertaining to lakes].

Represented by the aerobic strain MWH-EgelM2-3.acI, which was isolated from a small lake near Salzburg, Austria. Curved rods with lengths of 1.0–1.5 µm and diameters of 0.4–0.5 µm. No pure culture is available. Contained in a stable mixed culture together with 'Candidatus Limnoluna rubra' and a *Polynucleobacter necessarius* subsp. *asymbioticus* strain. The mixed culture grows well in liquid NSY medium. Supplementation of NSY medium with L-alanine results in an increase of the relative proportion of 'Ca. Planktophilia limnetica' by more than one order of magnitude. 'Ca. Planktophilia limnetica' can be recognized by the presence of the diagnostic oligonucleotide sequence 5'-GAAACTTGGTGGCATCRCCG-3' in the 16S rRNA gene (positions 183–193, *E. coli* numbering). Phylogenetic analysis of the 16S rRNA gene sequence indicated the affiliation of the *Candidatus* taxon with the order *Actinomycetales*.

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