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Ecophysiological characteristics of key members of *Betaproteobacteria* in freshwater bacterioplankton

Ph.D. Thesis

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Annotation

This thesis primarily focuses on one segment of freshwater *Betaproteobacteria*, the *Limnohabitans* genus (including the RBT lineage). As opposed to other recent research directions, the major aim was to recover the members of the previously uncultured RBT lineage through their isolation from various freshwater habitats. However, the results presented in this thesis have also ambitions to go far beyond the taxonomic descriptions only; the dissertation intends to contribute significantly to unveiling of important ecophysiological characteristics of the studied lineage in a set of both laboratory and field research. Therefore, understanding of growth characteristics, mortality, diversity and life strategies of aquatic microbes is of highest importance regarding profound human impact on water quality and increasing need of drinking water supplies.

Declaration [in Czech]

Prohlašuji, že svoji disertační práci jsem vypracoval samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

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Vojtěch Kasalický

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List of papers and author's contribution

The thesis is based on the following papers:

I. Hahn, M.W.; **Kasalický, V.;** Jezbera, J.; Brandt, U.; Jezberová, J. & Šimek, K. (2010) *Limnohabitans curvus* gen. nov., sp. nov., a planktonic bacterium isolated from a freshwater lake. Int J Syst Evol Microbiol 60: 1358–1365. (IF = 1.930)
VK was responsible for physiological tests, conducting of chemotaxonomic descriptions, comparisons of the strain with other species and revising the manuscript.

II. **Kasalický, V.;** Jezbera, J.; Šimek, K. & Hahn, M.W. (2010) *Limnohabitans planktonicus* sp. nov. and *Limnohabitans parvus* sp. nov., planktonic *Betaproteobacteria* isolated from a freshwater reservoir, and emended description of the genus *Limnohabitans*. Int J Syst Evol Microbiol 60: 2710–2714. (IF = 1.930)
VK isolated the described strains, run physiological tests, processed data on chemotaxonomic descriptions and DNA-DNA hybridizations and wrote the manuscript.

III. Hahn, M.W.; **Kasalický, V.;** Jezbera, J.; Brandt, U. & Šimek, K. (2010) *Limnohabitans australis* sp. nov., isolated from a freshwater pond, and emended description of the genus *Limnohabitans.* Int J Syst Evol Microbiol 60: 2946 – 2950. (IF = 1.930)
VK was responsible for physiological tests, for performing of chemotaxonomic descriptions, comparisons of the strain with other validly described species and participated on the manuscript revision.

IV. Šimek, K.; **Kasalický, V.;**, Jezbera, J.; Jezberová, J.; Hejzlar, J. & Hahn, M.W. (2010) Broad habitat range of the phylogenetically narrow R-BT065 cluster representing a core group of the betaproteobacterial genus *Limnohabitans*. Appl Environ Microbiol 76: 631-639. (IF = 3.778)
VK was responsible for the selection of habitats, sampling and determination of basic physical characteristics, and cell counting. He participated on the manuscript revision.

V. Šimek, K.; **Kasalický, V.;** Horňák, K.; Hahn, M.W. & Weinbauer, M.G. (2010) Assessing niche separation in coexisting *Limnohabitans* strains through interactions with a competitor, viruses, and a bacterivore. Appl Environ Microbiol 76: 1406–1416. (IF = 3.778)
VK was responsible for the complete preparation of the bacterial strains, including their pre-cultivation, for the virus concentration, lysogeny and plaque-forming tests, sampling and cell counting. He participated on the experiment designing and the manuscript revision.

VI. Šimek, K.; **Kasalický, V.;** Zapomělová, E. & Horňák, K. (2011) Algal-derived substrates select for distinct betaproteobacterial lineages and contribute to niche separation in *Limnohabitans* strains. Appl Environ Microbiol 77: 7307–7315. (IF = 3.778)
VK was responsible for the complete preparation of bacterial strains used, axenic culture collection, CARD-FISH, cell counting. He participated on the

manuscript revision.

VII. **Kasalický, V.;** Jezbera, J.; Hahn, M.W. & Šimek, K. Unveiling the diversity of the *Limnohabitans* genus, an important group of freshwater bacterioplankton, by characterization of 35 isolated strains. Submitted manuscript.
VK isolated 35 strains from the Limnohabitans *genus, run metabolic tests, was responsible for DNA extraction, PCR, sequence assembly, and phylogenetic analyses, and wrote the manuscript.*

Contents

1. Background rationale of the thesis

1.1. Historical milestones

The composition of pelagic microbial community has been the major objective of the aquatic research during the 80's of 20th century. The fluorescent nucleic acid-stains (acridine orange and DAPI) (e.g. Daley & Hobbie 1975, Porter & Feig 1980), together with good quality polycarbonate filters (Hobbie et al. 1977), epifluorescence microscopes, became revolutionary tools in study of microbes in water (reviewed by Kepner Jr. & Pratt 1994). Bacteria were described as the smallest but the most abundant organisms in aquatic habitats. Classical trophic cascade from algae via rotifers and zooplankton to fish has been changed incorporating of bacteria, heterotrophic nanoflagellates (HNF) and ciliates into so-called microbial loop (e.g. Azam et al. 1983). A common feature of the microbial community is a high turnover rate, including both rapid growth and mortality rates (Pace et al. 1990). Channelling of the dissolved organic matter (DOM) by microbes is of importance in all habitats, but it plays the key role particularly in places with high input of allochthonous DOM or in oligotrophic and dystrophic waters where the phytoplankton production is limited by nutrient or light availability, respectively (Tranvik 1990, Thomas et al. 1991). Moreover, shallow lakes with high amount of clay particles show only low autotrophic contribution to the zooplankton diet compared to the heterotrophic microbial web (Simon et al. 1992, Lind et al. 1997). Self-purification processes in the river part of reservoirs and lakes (Šimek et al. 1990), acidified lakes (Vrba et al. 1996), extremely acidic mines (Kamjunke et al. 2005) or traps of aquatic carnivorous plants (Sirová et al. 2010) are other examples with the main role of heterotrophic microbial communities.

At the beginning of the 21st century, the principle task was aimed at the description of the bacterial community composition (BCC, Glöckner et al. 2000) and key players identification on the level of family-/genus-like clusters (e.g. betI, Zwart et al. 2002, RBT, Šimek et al. 2001). It has been proved that the vast majority of bacteria, repeatedly occurring in both marine and freshwater habitats, is not phylogenetically related to any cultivated and/or validly described species (Glöckner et al. 2000). Moreover, even the first phylogenetic analyses of Zwart and colleagues (2002) showed that clusters of freshwater bacteria were paraphyletic, scattered among other non-

aquatic bacterial taxa. In addition to complex studies of BCC, significantly less studied metabolic activities *in situ* largely differed among individual lineages (Horňák et al. 2006, Salcher et al. 2008), indicating that the most abundant taxa are not the most active (Horňák et al. 2010, Salcher et al. 2010). The investigation on the impact of predation of small protists (HNF and ciliates) has revealed that protists select against the bacterial size (e.g. Šimek & Chrzanowski 1992, Hahn & Höfle 1999) and the specific features regarding the taxonomic affiliation (Šimek et al. 1997, Pernthaler et al. 2001). The virus infection has been recognized as an important mortality factor with a selective impact on the BCC, and also as a driving force for evolutionary change (Thingstadt et al. 1993, Weinbauer & Rassoulzadegan 2004).

Another break point of modern microbial ecology was the detection of high abundance of bacteriochlorophyll-containing bacteria, mostly aerobic anoxygenic photoheterotrophs (AAPs), in aerobic zone of oceans and freshwaters that changed views on physiological properties of common aquatic bacteria (Kolber et al. 2000, Mašín et al. 2008). Additionally, a large spectrum of photosensitive rhodopsines was determined in different bacterial clades (Sharma et al. 2009). Recently, in numerous German lakes, Salka with colleagues (2011) detected genes for bacterial photosynthesis affiliated to the vicinity of the *Rhodoferax* genus. Moreover, a high ability to survive the singlet oxygen stress was reported for *Limnohabitans* genotypes (Glaeser et al. 2010). Thus, the energetic balance of many heterotrophic aquatic bacteria is dependent on both DOM and the incident light (Koblížek et al. 2007, Gasol et al. 2008).

The overall knowledge on ecology and diversity of freshwater bacteria has been recently exhaustingly reviewed by Newton and colleagues (2011). Numerous black-boxes have been "opened" via linking of individual lineage groups with their role in freshwater habitats. Most likely, the used taxonomic units are too large to represent well defined and thus valid ecological units. However, the advance achieved during the last decade has changed the perception of bacterial diversity. Ecological features of ecotypes or genotypes are studied (Jezbera et al. 2011), or new narrow (restricted) phylogenetic lineages are incorporated into refined concepts and their interpretations (Eiler et al. 2012).

1.2. Culture-independent tool

The aquatic microbiology has noted a rapid progress in the development of molecular methods, highly efficient to study "uncultivable-microbes" (Muyzer et al. 1993, Alfreider et al. 1996, Rappé & Giovannoni 2003). Genetic distances between freshwater bacteria facilitated the design of probes targeting the key lineages and the rapid introduction of the fluorescence *in situ* hybridization (FISH, Glöckner et al. 2000) and more sensitive CARD-FISH (catalyzed reporter deposition, Sekar et al. 2003), which elegantly enabled to distinguish and count different bacterial groups in the whole community. Both methods are unlikely limited by the low variability of ribosome sequences, against which are the probes targeted. More sensitive DNA-based cultivation-independent tools, e.g. the denaturation gradient gel electrophoresis (DGGE, Burr et al. 2006), reverse-line blot hybridization (RLBH, Zwart et al. 2003), 454 pyrosequencing (Margulies et al. 2005) and Solexa sequencing (Illumina; Bennet 2004, Bentley 2006), were used to describe the genetic diversity. However, the high potential of molecular tools to recover species-/ecotype-/genotype-specific groups remains, dormant due to high ribosome sequence conservation compared to the high genome variability. Recently, a new tool called Single Cell Genome Analysis (SCGA) was introduced to overcome this problem (Stepanauskas & Sieracki 2007). This tool enables to determine not only the taxonomic affiliation of a bacterium but also its major genomic traits (Martinez-Garcia et al. 2012). This could be a powerful tool for the so far uncovered taxa known only from sequence data, or for taxa that could be cultivated only with other species (lineage Luna 2, *Actinobacteria*, Jezbera et al. 2009).

1.3. The importance of being "cultivated"

Nevertheless, the cultivation techniques have undergone an important progress as well. The most important innovations were coupled with the approaches such as gradual acclimation of bacteria to enhanced nutrient concentrations, filtration (Hahn et al. 2004a, 2009a), utilization of low DOM media (Watanabe et al. 2009) or signal compounds (Bruns et al. 2003), a shift to more frequent use of liquid media, number of replications (Gich et al. 2005) as well as employing of specific substrates (e.g. methane, Bussmann et al. 2006). While the uncovering of the microbial diversity has been mainly facilitated by molecular tools, the isolation plays an irreplaceable role in

the description of taxonomic units (species-ecotype-genotype), and also in the evaluation of their ecophysiological traits (cf. Eiler et al. 2012, Hahn et al. 2012a). Up-to-date, the correlation between 16S rRNA gene sequences variability and whole genomes differentiation has been found importantly humped on the species level (Stackebrandt & Ebers 2006, Fraser et al. 2009, Kämpfer and Glaeser 2012). Moreover, isolated strains create the basic interface for diversity description, specific markers development and their utilization (e.g. Hahn et al. 2005, Jezbera et al. 2011).

1.4. Ecological concepts of bacterial life strategies

Small cell size, high vulnerability to virus attack, and complexity and patchiness of a surrounding aquatic environment have been described as characteristics shaping pelagic lifestyles of particular bacterioplankton members. The traditional view on the ecological strategies was established on eukaryotic organisms as the representatives (Begon et al. 1990). As there are quite pronounced differences between bacteria and eukaryotes, this traditional view should be substantially revised to facilitate sound characterization of typical bacterial lifestyles (e.g. Logares 2011). The aquatic microbial ecology recognizes a number of life strategies to compete for limiting resources (uptake specialists) or to escape from being killed (predator defence specialists) (e.g. Thingstadt et al. 2005). Moreover, the virus impact on the prokaryote diversity and diversification is quite pronounced and can contribute even comparatively to overall bacterial loss rate as the protists-induced bacterial mortality (e.g. Weinbauer & Rassoulzadegan 2004). The dynamic stability and the ecological impact of predators or virus attack on bacterial community dynamics was explained by "Killing-the-winner" hypothesis, when the most successful species are highly attacked by grazers and/or viruses and are thus removed from the system (Thingstad & Lignell 1997). This was postulated as one of the major principles responsible for sustaining bacterial species diversity.

a) Size does matter

In the bacterial world, the cell size/volume has been proposed to play an important role in the life strategy adaptation (Young 2006). First, the most effective bacterial grazers are ~1.5 - 5 µm large (cell diameter) heterotrophic nanoflagellates (HNF) selecting for the prey size within a range of 0.5 - 2 µm (Šimek & Chrzanowski 1992). Second, the

bacterial size seems to be related to the genome size and consequently to the physiological versatility of a bacterium, so-called "metabolic IQ" (Galperin 2005, Yooseph et al. 2010). However, this hypothesis is in contradiction with a described genome divergence of two *Prochlorococcus* ecotypes (Rocap et al. 2003), where the ecotype with a larger genome size was reported from more stable nutrient conditions.

b) Shaping a bacterium

Bacterial shapes are determined genetically, basically by presence (in rods) or absence (in cocci) of actin-like and tubulin-like proteins (Margolin 2009). It was suggested that rod-like bacteria have arisen first and coccoid forms being derivatives at the end of evolutionary lines (Siefert & Fox 1998). In contrast, there is only a little known about the genetic differentiation between solenoids and rods. Intermediate filament proteins (as crescentin in *Caulobacter*) are candidates of being responsible for the typical vibrioid shape of a bacterium (Cabeen & Jacobs-Wagner 2007, but cf. Dye et al. 2011). The evolutionary relationships of rods and solenoids/vibrioids are not clear and evidences from other species are rare (Siefert & Fox 1998, Bagchi et al. 2008). It is possible that many single-cell bacteria spend their life as prisoners of their shapes, i.e. the solenoid bacteria can not optimize their "area to volume" ratio and become cocci, thus the genome evolution (and streamlining) is tightly associated with their morphology. Consequently, the solenoid bacteria may have different demand (cell quota) of N and/or P compared to cocci with a similar volume.

c) Bacterial opportunism

Aquatic uptake specialists (opportunists, r-selection) are characterized by high growth rate under conditions of high resource availability, and high predation vulnerability. It has been proposed that they could exhibit the so-called "feast-or-famine" lifestyle (Yooseph et al. 2010), an adaptation to short and intensive nutrient pulses (e.g. photosynthetic exudates) and long starvation. Bacteria could exploit another type of survival strategy called a “Winnie-the-Pooh-strategy” (Pernthaler 2005, Thingstad et al. 2005) - maximizing uptake and predator defence (e.g. by increasing cell size) simultaneously.

d) Defence specialization

In contrast, a number of diverse strategies to escape the grazing pressure have been recognized for the defence-specialists among bacteria (Jürgens and Matz 2002).

Exopolymer synthesis, filament or aggregate formation fulfil all prerequisites for the K-selection (for stable environments), with preferential energy investment into their biomass (Hahn et al. 1999, 2000, 2004b, but cf. Wu et al. 2004). In contrast, S-selection (for stress) with lower growth rates but higher substrate affinities (Begon et al. 1990) represents a more suitable life strategy definition for planktonic ultramicrobacteria. Such a life strategy was proposed for planktonic *Actinobacteria* from the Luna 2 cluster, which, in addition to the small size, profit from their cell wall constitution (Tarao et al. 2009). Most recently, genome comparisons of bacteria from marine 'Candidatus *Pelagibacter'* genus (SAR11 cluster) (Yooseph et al. 2010) and freshwater *Polynucleobacter* genus (Hahn et al. 2012a) have suggested a "cryptic escape" strategy, a passive lifestyle surprisingly resulting in a large abundance of species with decreased vulnerability to protistan predation. These bacteria are characterized by a small genome size (related to the genome streamlining), a relatively small number of genes involved in the transduction of environmental signals, and the lack of motility and quorum sensing.

1.5. Diversity and evolution of freshwater *Betaproteobacteria*

The freshwater members of *Betaproteobacteria* can be currently subdivided into 18 lineages proposed as deepest branches of the genus/species taxonomic position (Newton et al. 2011, Figure 1). Although some names pre-define their differentiation and basic metabolism (such as *Methylophillus*, Garrity et al. 2005), their phylogeny is not supported by any physiological trait (see Box 1, Cavalier-Smith 2010). The most studied members, *Polynucleobacter* and *Limnohabitans* genera, belong into two large families, *Burkholderiaceae* and *Comamonadaceae*, respectively, harbouring bacteria with a broad spectrum of their lifestyles including symbionts, pathogens and free-living forms (Garrity et al. 2005). As a result, both parent segments do not bring to their descendants any clear characteristic (but see Box 2). Moreover, nothing is known about the genetic events (extras or loss) that differentiated freshwater clades from their phylogenetic neighbours (Logares et al. 2009). However, experiments conducted during the last ten years have documented significant ecological differences between both genera (e.g. Salcher et al. 2008, Jezbera et al. 2012). Bacteria of the *Limnohabitans* genus mostly exhibited the opportunistic strategy (Šimek et al. 2006),

while many *Polynucleobacter* genus members exploited the cryptic strategy (Hahn et al. 2012a).

Box 1 - Ecological evolution of Bacteria

The diverse metabolism enables the Bacteria to live in various habitats and inquires a question on mechanisms of their evolution. Interestingly, freshwater lineages differ phylogenetically from both marine and terrestrial bacteria (Glöckner et al. 2000, Zwart et al. 2002, Newton et al. 2011), but such delineation does not reflect the phylogeny. It seems possible that the diversification of bacteria inhabiting these three distinct habitats has started after massive metabolic diversification about 2.5 Gyr ago with oxygenic photosynthesis which yielded a huge diversity of chemotrophic and heterotrophic negibacteria (= Gram-negative, Cavalier-Smith 2010). Consistently with this theory, *Actinobacteria* probably emerged even later, about 1.5 Gyr ago. Eukaryotic primary producers, as well as grazers, emerged about 1 Gyr, that coincides with the genus/species diversification. One of the biggest invention in negibacteria is the utilization of ubiquinones in respiration pathways by *Alpha-*, *Beta-* and *Gammaproteobacteria* (Nowicka & Kruk 2010). Ubiquinones are able to use the oxygen as the terminal electron acceptor, which results in higher energy gain (Søballe & Pool 1999). The absence of *Betaproteobacteria* in almost all pelagic marine samples (Zwart et al. 2002, DeLong et al. 2006) indicate that they all have lost the ability to survive in higher salt concentrations or - in other words - they evolved by loosing this potential. The data about the presence of some *Rhodoferax* and *Limnohabitans* bacteria in Baltic sea or in brackish waters (Zwart et al. 2002, Shaw et al. 2008, K. Piwosz personal communication) could support the latter conclusion about possible mutation related to the decreased salinity tolerance.

It has been recently recognized that the *Polynucleobacter* genus is much more diversified than one would expect from its rather unified morphology, 16S rDNA and intergenic spacer (IGS) phylogenies and distribution data (Hahn et al. 2005, 2009b, 2010, 2011ab, Jezberová et al. 2010). Similarly, existence of large number of *Limnohabitans* sequences (more than 700) in databases suggests its further intragenous diversification. This fact sets an intriguing question on ecological principles of the evolutionary processes on lower than the genus level. Three known examples, *Prochlorococcus*, *Polynucleobacter* and *Vibrio*, indicate that environmental gradients (Johnson et al. 2006), physiochemical factors (Jezbera et al. 2011), seasonality and particle-associations (microhabitats) (Hunt et al. 2008) can be the relevant drivers of a diversification. The existence of microbial partitioning within the water column requires a new way to understand bacterial ecology, evolution and functions within the non-uniform and highly "patchy" aquatic environments (Grossart & Tang 2010).

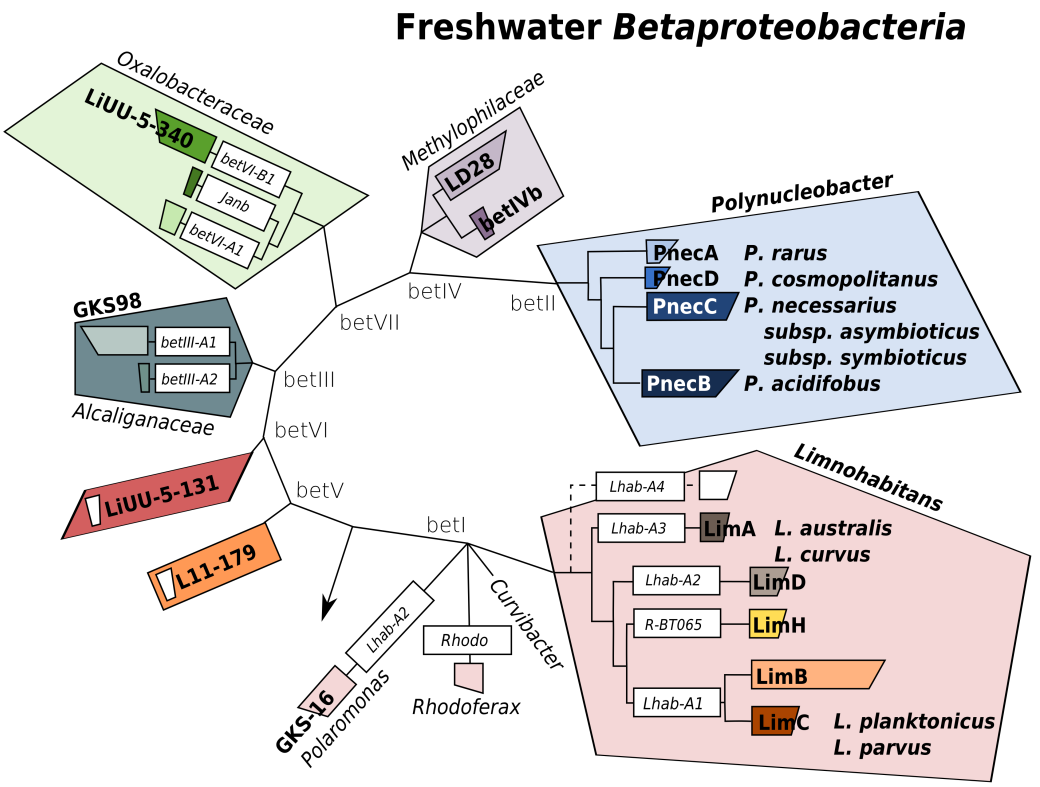

Figure 1
Diversity of freshwater *Betaproteobacteria* with detailed focus on *Limnohabitans* and *Polynucleobacter* genera. Tribes betI-VII according to Zwart et al. (2002) and Newton et al. (2011). White rectangles show the lineage/tribe names suggested by Newton et al. (2011). Size of coloured trapezoids represents mount of affiliated sequences in databases. Uncertain affiliation of Lhab-A4 to the *Limnohabitans* genus is stressed. Based on above mentioned publications and modified according the results in Hahn et al. (2012b) and manuscript 7. See also discussion in manuscript 7.

1.6. My address is "Everywhere"

The sequences affiliated with the *Limnohabitans* genus have been found in freshwater habitats of at least three continents (Glöckner et al. 2000, Zwart et al. 2002, Page et al. 2004, see Figure 5 in manuscript VII). Habitats included all types of environments, from oligotrophic (Lake Gatun in Panama, Shaw et al. 2008) to hypertrophic lakes (Wuliangsuhai Lake in China, Feng et al. unpublished), both arctic (Toolik Lake in Alaska, Crump et al. 2003) and tropic climates (mangrove in Taiwan, Liao et al. 2007), high mountains (Alpine Lake Joeri XIII in Switzerland, Yuhana unpublished) and lowlands (Římov Reservoir, Czech Republic, Šimek et al. 2001). In addition to stagnant habitats, the *Limnohabitans* OTUs were retrieved from lower parts of rivers

(Parker and Ipswich rivers in USA, Zwart et al. 2002, Crump & Hobbie 2005) as well as from brackish waters (Delaware and Chesapeake Bays in USA, Shaw et al. 2008). The above findings suggest that there are no geographical limits for the worldwide distribution of *Limnohabitans* bacteria.

Box 2 - Ecological evolution of aquatic *Betaproteobacteria*

The members of freshwater *Betaproteobacteria* are often affiliated with *Burkholderiaceae*, *Comamonadaceae*, *Methylophilaceae*, *Oxalobacteraceae* and *Alcaligenaceae* families (Zwart et al. 2002). Whereas the specialization and basic metabolism of several families is emphasized (Brenner et al. 2001), the phylogeny of two most important (*Burkholderiaceae* and *Comamonadaceae*) is not supported by any physiological trait (Cavaler-Smith 2010). Nevertheless, the so far cultivated members of the *Comamonadaceae* family possess a unique chemotaxonomic trait, the presence of 3-hydroxyoctanoic acid ($C_{8:0}$ 3-OH). 3-hydroxy fatty acids are important components of lipopolysaccharides and of the lipid A, bacterial endotoxin, where their number, nature and distribution are responsible of the lipid A bioactivity (Leone et al. 2007). Moreover, the short-chained and branched fatty acids are more abundant at lower temperatures for preservation of membrane fluidity than long and unbranched ones (Sinesky 1974, Mrozik 2004). The *de-novo* synthesis of short-chained fatty acids in the family *Comamonadaceae* requires less energy than synthesis of fatty acids with longer chains, which could result in higher growth potential of these bacteria. Next, 2-hydroxy fatty acids seem to be absent in *Comamonadaceae*, with the only exception being *Variovorax paradoxus* (Willems et al. 1991, Urakami et al. 1995). Their role in ecophysiology of freshwater bacteria has not been clarified yet. 2-hydroxy fatty acids are incorporated into polar lipids (e.g. phospholipids) which are essential membrane components of living cells, and they also contribute to molecular stress signalling in sphingolipids in intestinal *Bacteroides* (An et al. 2011). The production and specific incorporation of 2-hydroxy fatty acids into phosphatidylethanolamines, ornithine amide lipids increase as the growth temperature rises in *Burkholderia* (Taylor et al. 1998). The 2-hydroxy groups of fatty acids could thus be involved in stabilization of bacterial membranes at high temperatures.

Moreover, the presence of *Limnohabitans* bacteria was reported from two distinct freshwater invertebrates - the epithelium of *Hydra vulgaris* from Pohlsee (Fraune & Bosch 2007) and the digestive tract of *Daphnia magna* (Freese & Schink 2011).

FISH investigations revealed that R-BT065 probe (Šimek et al. 2001) targeted bacteria inhabit the whole vertical profile of a certain habitat - the neuston (Hörtnagl et al. 2010), the epilimnion (e.g. Šimek et al. 2001), and also the hypolimnion (Buck et al. 2009, Salcher et al. 2011). Additionally, they confirmed the presence of the RBT lineage in high mountains lakes (Pérez & Sommaruga 2007), and of course in reservoirs and ponds (Salcher et al. 2007). RLBH investigations strongly supported the presence of bacteria targeted with Rho-BAL47 probe (for betaI cluster) in arctic habitats (Zwart et al. 2003).

The importance of the particle-associated bacteria has been overlooked for a longtime because of their relatively low abundances (e.g. Unanue et al. 1992, Grossart & Simon 1993, Turley & Stutt 2000). This view has been revised since the specific activities of particle-bound bacteria were found to be of greater importance than those of the free-living ones (Grossart et al. 2007). It was proved that bacteria-attached to particles, phytoplankton or zooplankton differ phylogenetically from the free-living ones (DeLong et al. 1993, Acinas et al. 1999, Crump et al. 1999, Fandino et al. 2001). The *Limnohabitans* bacteria have not been described as particle-associated yet and the Rho-BAL47 cluster was not detected on particles larger than 20 µm (Zwart et al. 2003). However, the presence of related beta I genotypes has been detected in such environment (Parveen et al. 2011). This study also reported a complete lack of the RBT lineage in the 99 OTUs for beta I clade in Lake Bourget in France by RFLP. However, the reason of their absence remains unknown and is in contradiction with the vast majority of available literature data referred above.

1.7. Major substrate sources for bacterioplankton

In aquatic ecosystems, the organic matter used by heterotrophic bacteria is traditionally divided according to its form (dissolved vs. particulate) and origin (allochthonous vs. autochthonous). It is a mixture of variable substances whose qualitative and quantitative composition can not be clearly characterized (Lampert & Sommer 2007, cf. Woods et al. 2010). Therefore, the current research of investigations is focused on monomeric substances that contribute to only small fractions of DOC, but also on the photodegradation of highly complex allochthonous DOC including humic acids (Tranvik 1988, Jansson et al. 2007).

Uptake of radioactively-labelled substrates is a powerful and sensitive tool to study the assimilation of individual DOC entities by bacterioplankton community (e.g. Teira et al. 2004). The RBT lineage members are characterized (via MAR-FISH) by a high percentage of cells incorporating leucine (Horňák et al. 2006, Salcher et al. 2008, Buck et al. 2009, Pérez et al. 2010) and glucose (Buck et al. 2009), whereas low uptake rates were measured for thymidine (Horňák et al. 2006, Pérez et al. 2010) and acetate (Buck et al. 2009), and no uptake was detected for the 4-hydroxybenzoic acid (Buck et al. 2009). Other tools, such as substrate-induced-respiration profiles (Sinsabaugh &

Foreman 2001) or enzymes activity patterns (Sinsabaugh et al. 1997) provide information on the bulk bacterial community characteristics that can not be easily attributed to the individual RBT group/lineage members.

Limnohabitans bacteria can actively live (incorporate glucose and leucine) in both oxic and anoxic environments (Buck et al. 2009). Moreover, the RBT lineage of the *Limnohabitans* genus was described as a fast growing bacterioplankton segment (Šimek et al. 2001, 2006) and it was proposed that their population dynamics is coupled to the release of phytoplankton exudates (Šimek et al. 2008). The presence of certain blue-green algae, however, seems to inhibit their growth (Eiler & Bertilsson 2004, Horňák et al. 2008). Regarding the knowledge gained, it has been proposed that the *Limnohabitans* bacteria possess an opportunistic life strategy (Šimek et al. 2005, 2008, Salcher et al. 2007).

Thus, it has become more and more obvious that the current oversimplified concepts of bacterial lifestyles (e.g. Thingstad et al. 2005) should be revised carefully (cf. Grossart 2010) in the light of novel findings of life and genomic traits of abundant groups of bacterioplankton (e.g. Yooseph et al. 2010, Dupont et al. 2011, Hahn et al. 2012a). Overall, however, the metabolome of the *Limnohabitans* bacteria is most likely preconditioned on the use of the highly energetic substrates (cf. Buck et al. 2009). Currently ongoing genome sequencing *of L. planktonicus* will definitely bring new insights into the debate about major bacterial life strategies in plankton.

2. Hypotheses and Objectives

2.1. *Highly abundant but uncultivated members of freshwater bacterioplankton could be isolated from freshwater habitats by a modification of available methods.*

Isolation of strains from the RBT lineage by modification of the FAM method, and the taxonomic definition of these bacteria.

2.2. *Bacteria from the* Limnohabitans *genus (i.e. RBT lineage) seem to be opportunistic strategists, likely adapting to "feast-or-famine lifestyle". Their success in freshwater habitats is related to their metabolic versatility and high growth potential that obviously override the effect of the intense protistan grazing pressure, which members of the RBT lineage experience in plankton environments.*

Metabolic versatility will be studied by utilization of simple substrates, as well as under different temperature and salinity concentrations. Protistan grazing will be investigated employing an axenic *Poterioochromonas* culture and natural HNF communities.

2.3. *Population dynamics of* Limnohabitans *bacteria is related to the key environmental drivers such as habitat pH and DOM characteristics as the allo-/autochthonous organic matter production, and mainly to algal exudation rates.*

Presence and abundance of the bacteria targeted by the R-BT065 probe will be determined by FISH in habitats with different limnological characteristics.

2.4. *The microdiversity within the* Limnohabitans *genus is much larger than one could expect from the 16S rRNA genes similarities. Thus, novel markers should be designed and applied to unveil its diversity.*

Morphological and physiological comparison of strains isolated from different habitats will be performed. Intergenic spacer between ribosomal genes will be tested as a potential new marker.

3. Results and Discussion

3.1. Summary of the most important results

The crucial contribution of this thesis to the current microbial ecology is the isolation of bacterial strains from the *Limnohabitans* genus, mainly from its RBT lineage and characterization of their ecophysiological capabilities (**paper II, manuscript VII**). At the moment, our collection contains more than 40 viable strains isolated from diverse environments. Data on validly described species (*L. australis*, *L. curvus*, *L. parvus* and *L. planktonicus*) are deposited in public collections (DSMZ, Institute Pasteur), and thus can be further studied by the scientific community. The successful isolation of the members of one of the key bacterioplankton group (cf. **paper IV**) contributed quite significantly to the research that is entirely dependent on the isolated strains (e.g. **papers V**, **VI**, Blom et al. 2011, Horňák et al. 2012, Šimek and Kasalický, unpublished data), and on which I participated as a core collaborator. Notably, these internationally sound results initiated new cooperations with researchers from Institute of Microbiology ASCR in Třeboň (Michal Koblížek), University of Zürich (Judith Blom), Justus-Liebig-University, Giessen (Jens Glaeser) and University of Konstanz (Heike Freese).

3.2. Methods innovation

a) Modifications of the existing isolation methods

Modified "Filtration and acclimation method" (Hahn et al. 2004a) was found as the most suitable tool for isolation of *Limnohabitans* strains (**papers I, II, III, manuscript VII**). The modification is based on separation of bacteria from grazers using 0.8 μm pore-size filter, followed by the overnight activation in the water from their home environment and the subsequent dilution to extinction ("Separation, activation, dilution and acclimation method" - SADAM). However, SADAM was not efficient for isolation of bacteria from humic habitats (cf. *Polynucleobacter* recovery by Hahn et al. 2005), neither from high mountain lakes as Gossenköhlesee. Therefore, I have to admit that the still uncultured phylotypes could represent an important part of diversity (Figure 2).

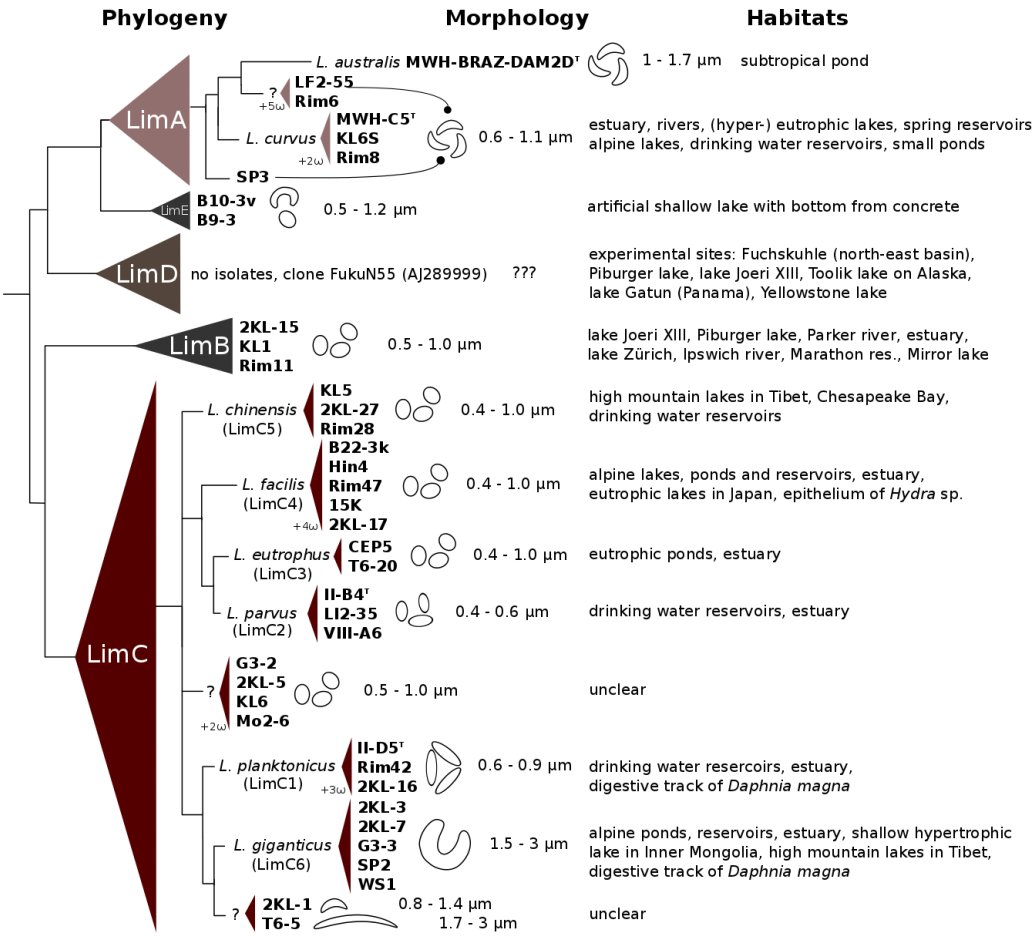

Figure 2
Microdiversity of *Limnohabitans* genus based on 40 isolated strains. The simplified phylogeny schema was build on analyses of 16S rRNA gene and IGS1 sequences as presented in manuscript VII. Symbol "ω" with a number stands as reference for isolated strains obtained by K. Watanabe. New names for members of coherent lineages are proposed. Question marks stands for polyphyletic groups of strains with similar morphologies. Listed habitats originate from GenBank, EMBL sequence databases.

b) Newly proposed phylogenetic markers

The low genetic diversity indicated by the previous studies (Zwart et al. 2002, Newton et al. 2011) using the ribosomal SSU gene highly contrasts with the surprisingly rich morphologies and different patterns in substrate utilization of isolated strains (**manuscript VII**). Therefore, the intergenic spacer between 16S and 23S rRNA genes (IGS) was successfully tested as a fine-scale marker to delineate individual lineages and even the genotypes. The IGS marker was already used for delineation of *Polynucleobacter* sublineages (Hahn et al. 2005), and it was proved to be an indispensable tool for unraveling the specific ecotypes, i.e. narrow genotype groups

with contrasting habitat preferences (Jezbera et al. 2011).

c) Modifications of experimental designs

A new experimental design was proposed to conduct ecological investigations of isolated strains in predator-prey-competitor systems, further manipulated by an environmental virus concentrate (**paper V**). The design combined axenic cultures (bacteria or protists), together or with a natural virus concentrate collected from the Římov Reservoir, from which the *Limnohabitans* strains were isolated. The design enabled comparing the fitness of even closely related strains, and their vulnerability to mortality factors represented by (i) an axenic culture of *Poterioochromonas* sp.; combined with (ii) different doses of live or heat-killed virus concentrate; both mimicking predator and virus concentrations present at *in situ* conditions. Additional modified experiments proved the potential of this method for estimating of HNF growth parameters and net growth yield on the representative strains of *Limnohabitans* but also other bacterial isolates that were offered as a dominant prey for HNF (Šimek et al. unpublished data).

3.3. *Limnohabitans* bacteria and their typical habitats

Aquatic habitats are often classified according to their trophic status (i.e. different concentrations of dissolved phosphorus and chlorophyll *a* content; Lampert & Sommer 2007). However, the distribution of each organism could be driven by its specific requirements. We have defined such requirements for the whole RBT lineage (bacteria targeted by the R-BT065 probe) of the *Limnohabitans* genus (**paper IV**). The relative abundance and absolute abundance of these bacteria were significantly and positively related to higher pH, conductivity and the proportion of low-molecular-weight compounds in DOC, and negatively related to the total DOC and dissolved aromatic carbon contents. The pH is probably one of the most important factor in the habitat selection throughout the major freshwater bacterial clades (Lindström et al. 2005, Hahn 2006). The pH represents an easily determinable value for many chemiosmotical factors in a solution (e.g. availability of nutrients, predominant ionic forms of metals and their toxicity). Recently, a major role of pH has been recognized in the ecological diversification of *Actinobacterial* freshwater lineages (Newton et al. 2007) and *Polynucleobacter* genus (Jezbera et al. 2011, Hahn et al. 2012a).

The unveiled microdiversity of the *Limnohabitans* genus (**manuscript VII**) has questioned whether its widespread distribution was achieved by multiple or by a single ecotype/genotype group colonization. Similar task was investigated for *Polynucleobacter necessarius* subsp. *asymbioticus* by Jezbera and co-workers (2011), revealing the mosaic distribution of individual genotype groups or even their complete niche separation. Its ubiquity thus results from the ecological diversification within the taxon and not from generalist adaptation of strains (Jezbera et al. 2011). Very similar situation could be expected in *Limnohabitans* genus, because environmental clone libraries with *Limnohabitans*-affiliated sequences indicate a broad distribution of individual lineages, as well as the presence of multiple genotypes within a single water sample (**paper II, manuscript VII**).

The plausible explanation is an existence of microhabitats (i.e. patchy character of plankton, presence of organic particles, algae, cyanobacteria, protists, zooplankton associations etc.) selectively occupied by individual bacterial genotypes (Riemann & Winding 2001, Boenigk & Arndt 2002, Simon et al. 2002, Grossart et al. 2003, Eiler et al. 2006). The particle-like structures are important hot-spots of bacterial activity in oligotrophic pelagic waters, while interspecific networks (an addiction on different organisms) are present in all types of habitats (Grossart 2010). The latter case is probably of importance for *Limnohabitans* genus members (**papers V**, **VI**, Freese & Schink 2011). Since a planktonic bacterium could have more complex lifestyle than generally believed, individual complex “bio-habitats” or “interactive-habitats” (defined with presence/absence and interaction of another, e.g. algal species) are of highest importance to understand their ecology (Grossart & Tang 2010).

3.4. Predator-prey interactions

The predator-prey relationship is one of the best studied microbial interactions in microbial ecology. The members of the *Limnohabitans* genus were found to be highly vulnerable to HNF predation *in situ* (Šimek et al. 2001, 2006, Jezbera et al. 2005) and to the *Poterioochromonas* predation in a specifically designed laboratory experiment (**paper V**). The presence of predators, live viruses, and a competitor (*Limnohabitans* versus other bacterial groups, e.g. *Flectobacillus*) significantly affected their population dynamics in the experimentally manipulated treatments. In fact, *L. parvus*

profited from the grazing on its larger competitor – the *Flectobacillus*, while *L. planktonicus* had advantage from being virus-resistant. Notably, the success of the RBT lineage members is congruent with results from *in situ* manipulations (Šimek et al. 2005, Jezbera et al. 2006), clearly documenting the large growth potential of this lineage. Moreover, recent experimental analysis revealed that *Limnohabitans* members support faster HNF growth and contribute significantly more to the HNF biomass production than e.g. *Polynucleobacter* or mainly *Actinobacteria* members (Šimek and Kasalický, unpublished data). The vulnerability of *Limnohabitans* bacteria to protist predation is probably a trait shared within the genus, or at least within the RBT lineage.

Bacteria exhibit various defence mechanisms against grazers and predators (Hahn et al. 1999, Pernthaler et al. 2005). Tested strains of *Limnohabitans* genus seem to lack such an adaptation. A volume increase during the induction of the growth is typical for all *Limnohabitans* strains (Kasalický, personal observations). For the isolated strains used for grazing experiments - *L. parvus*, *L. planktonicus*, strains 2KL-1, 2KL-27, Rim 11, Rim 28, 15K, we have never observed a morphological shift induced by the predator presence, like *Flectobacillus* filamentation or *Sphingomonas* clustering (**paper V**, Blom et al. 2010, Šimek and Kasalický, unpublished data).

The genus *Limnohabitans* includes bacteria of variable size and morphology (**papers I, II, III, manuscript VII**). While grazing pressure of HNF may remove larger bacterial cells, the smaller are persisting (Šimek and Chrzanowski 1992, cf. Figure 2 Young 2006). Pernthaler (2005) proposed in his review that there was no other convincing explanation why to be an ultramicrobacterium than the loss-rate reduction (but cf. Yooseph et al. 2010 and Hahn et al. 2012a). Experiments on the predation of small-sized *Limnohabitans* strains and predation of similar sized *Polynucleobacter* and *Actinobacteria* strains suggest that not only the size, but also the taxa-specific traits play the key role in the bacterial strain loss-rates and the biomass yield of HNF (Šimek and Kasalický, unpublished results). It is conceivable that the advantage of a small size plays a role only on the genus-level, whereas properties of bacterial cell wall, cell surface charge, hydrophobicity, stoichiometry, extracellular polymeric substances or other unknown mechanisms influence grazing loss-rates of bacteria from different tribes (Monger et al. 1999, Matz & Jürgens 2001, Matz et al. 2004, Pernthaler 2005). In addition, there is no reasonable explanation whether the curved/solenoid

morphology, frequently found in freshwater bacterioplankton (Posch et al. 2009), brings any advantage to bacteria regarding their grazing protection. The study of *Limnohabitans* strains (**manuscript VII**) can bring novel insights into this topic. The fact that strains with different morphologies are affiliated within one genus enables an interpretation of the strain-specific predation vulnerability (even as to size non-related defence strategy), resembling e.g. currently running process of species differentiation as suggested for the *Polynucleobacter* sp. (Jezbera et al. 2011).

3.5. Metabolical variability and bottom-up control

The MAR-FISH investigations revealed group-specific differences between freshwater planktonic bacteria at *in situ* studies of uptake of sugars and amino acids (Horňák et al. 2006, Salcher et al. 2008, 2011, Buck et al. 2009, Pérez et al. 2010). High degree of metabolical variability was found within closely related *Limnohabitans* strains under laboratory conditions (**papers I, II, III, manuscript VII**). These results undoubtedly contribute to the ongoing debate on the physiological diversification, however their interpretation in the context of microbial ecology is not straightforward. The reactive pool of DOM/DOC, representing the most common carbon sources available for bacteria, is composed of relatively few group elements such as carbohydrates, amino acids, fatty acids, hydrocarbons and steroids (Benner et al. 1992). However, a deeper insight into their characterization is very limited because the available tools mostly do not provide any information on their structure and physiochemical properties (Lam et al. 2007). To date, there is only a limited knowledge on the source and origin of individual substrates in the freshwater habitats (Giroldo et al. 2005, Jansson et al. 2007). Moreover, the difficulties to link bacterial genes, source and quality of substrates complicate the description of many interspecific relationships of microorganisms in aquatic habitats (Grossart & Tang 2010).

Algae are known as important autochtonous source of organic matter for bacteria (Baines & Pace 1991). Bacteria of the *Limnohabitans* genus were found to be tightly connected to phytoplankton dynamics and exudation rates (**paper VI**). They were present in high numbers in established cultures of green algae and cryptophytes, whereas their contribution was low in *Cyanobacterial* cultures. Moreover, two *Limnohabitans* species produced significantly higher biomass yield when co-cultured

with axenic strains of *Cryptomonas*, *Chlamydomonas* and *Pediastrum* sp. The highest biomass yield was determined with *Cryptomonas* algae which was already suggested from earlier observations in the Římov Reservoir where the abundance of the RBT lineage correlated with exudation rates and cryptophytes dynamics (Šimek et al. 2008). On the contrary, *Limnohabitans* bacteria seem to be rather negatively affected by presence of *Cyanobacteria* in both laboratory and field studies (**paper VI,** Horňák et al. 2006, 2008, 2012, Shi et al. 2009, Li et al. 2011 but cf. Eiler & Bertillson 2004). Their presence was not reported from benthic diatom assemblages (Bruckner et al. 2008).

3.6. Niche separation of two *Limnohabitans* species

Apart from the species description based on physiological and genetic features, it is of high importance to search for valuable ecological aspects that could differentiate between two species (Hahn et al. 2012a). As the highest competition is expected between the closely related organisms (Lampert & Sommer 2007), the more competitive (but highly grazed) strain should be theoretically replaced by a closely related strain rather than by a strain from another bacterial group.

We have proposed the separation of ecological niches for two closely related *Limnohabitans* species, *L. parvus* and *L. planktonicus*, regarding the relationship to both top-down and bottom-up controlling factors. The vulnerability to *Poterioochromonas* sp. grazing together with high growth rate, competition fitness (**paper V**) and the metabolic abilities (**paper II**) were suggested to be higher for *L. planktonicus*. On the contrary, *L. parvus* was more vulnerable to the virus attack (**paper V**), while *L. planktonicus* grew better in co-cultures with *Cryptomonas*, *Pediastrum* and *Chlamydomonas* sp. (**paper VI**). Thus, ecological niches characterizing the strains of *L. parvus* and *L. planktonicus* differ markedly in their growth potential and vulnerability to mortality factors, i.e. grazing and virus lysis. Hence, these two species will undergo genome analysis, and important insights justifying these differences are likely to be revealed. Because the both examined *Limnohabitans* species (**paper II**) originated from the same habitat, it is probable that they could represent suitable model organisms for testing hypotheses about niche separation within a particular habitat.

4. Conclusions and further perspectives

The isolation focus of my thesis resulted in description of the *Limnohabitans* genus - a missing taxonomic unit for the betaI, Rho-BAL47 clusters (Zwart et al. 2002). Moreover, the description of four new species and the established collection of bacterial strains unveiled the microdiversity in the RBT lineage (Šimek et al. 2001) and in the recently defined groups Lhab-A1 to A4 (Newton et al. 2011). We documented the pH along with conductivity and low molecular weight DOM as main factors determining the presence and abundance of bacteria from the RBT lineage. Moreover, we described the potential of the algal derived (autochthonous) DOM as the sole carbon source for growth of two isolated strains. None of the concentration of main nutrients (phosphates and nitrates) played a significant role in the (relative) abundance of RBT lineage bacteria. We proved a niche separation of two *Limnohabitans* strains coexisting in the same habitat based on predation vulnerability, competitiveness and resistance to virus attack. The separation was evident also from metabolic traits and different growth rates in co-cultures with algae. Large metabolic versatility has been found within strains from the *Limnohabitans* genus, however, exceptions are present. Due to the morphological traits and phylogeny of isolated strains, we proposed new species-like lineages within the *Limnohabitans* genus. As first, we established IGS markers to better characterize the microdiversity of the genus.

Currently, two genera of freshwater *Betaproteobacteria* - *Polynucleobacter* and *Limnohabitans* - have all prerequisites (isolated members, considerable knowledge on their ecology and microdiversity) to become the model organisms for further studies of microbial ecology and microbial networks in freshwater habitats. Such a model system allows to solve the puzzle of unknown genomic traits, species diversification, environmental variables and interactions. Most recent approaches suggest a complex web structure of microbial interdependencies in aquatic systems (Eiler et al. 2012). However, there is still a lack of the microdiversity description and of the definition of relevant taxa.

I hope that information and knowledge accumulated during my Ph.D. study have a large potential to contribute significantly in the further research on freshwater bacteria.

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Paper no. 1

Hahn MW, **Kasalický V,** Jezbera J, Brandt U, Jezberová J & Šimek K (2010) *Limnohabitans curvus* gen. nov., sp. nov., a planktonic bacterium isolated from a freshwater lake. *Int J Syst Evol Microbiol* **60:** 1358-1365.

(IF = 1.930)

Abstract

A chemo-organotrophic, aerobic, facultatively anaerobic, non-motile strain, MWH-C5^T, isolated from the water column of the oligomesotrophic Lake Mondsee (Austria), was characterized phenotypically, phylogenetically and chemotaxonomically. The predominant fatty acids of the strain were $C_{16:1}\omega7c/\omega6c$, $C_{16:0}$, $C_{12:1}$ and $C_{8:0}$-3OH, the major quinone was ubiquinone Q-8 and the G+C content of the DNA of the strain was 55.5 mol%. 16S rRNA gene similarity to the closest related type strains was 96.6 % (*Curvibacter delicatus* LMG 4328^T) and 95.7 % (*Rhodoferax fermentans* FR3^T). Phylogenetic analysis of 16S rRNA gene sequences revealed the affiliation of the strain with the family *Comamonadaceae* (*Betaproteobacteria*); however, the phylogenetic position of the strain did not support an affiliation to any previously described genus within this family. A family-wide comparison of traits revealed that the strain possesses a unique combination of DNA G+C content, major fatty acids and major 3-hydroxy fatty acid. Furthermore, the strain differs in several traits from the closest related genera. Based on the phylogeny of the strain and differences from closely related genera, we propose to establish the new genus and species *Limnohabitans curvus* gen. nov., sp. nov. to accommodate this strain. The type strain of *Limnohabitans curvus* is MWH-C5^T (=DSM 21645^T =CCUG 56720^T). The type strain is closely related to a large number of uncultured bacteria detected by cultivation-independent methods in various freshwater systems.

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DOI: 10.1099/ijs.0.013292-0

Paper no. 2

Kasalický V, Jezbera J, Šimek K & Hahn MW (2010) *Limnohabitans planktonicus* sp. nov. and *Limnohabitans parvus* sp. nov., planktonic *Betaproteobacteria* isolated from a freshwater reservoir, and emended description of the genus *Limnohabitans. Int J Syst Evol Microbiol* **60:** 2710 – 2714.

(IF = 1.930)

Abstract

Two bacterial strains, II-B4^T and II-D5^T, isolated from the meso-eutrophic freshwater Římov reservoir (Czech Republic), were characterized phenotypically, phylogenetically and chemotaxonomically. Both strains were chemo-organotrophic, facultatively anaerobic, non-motile rods, with identical DNA G+C contents of 59.9 mol %. Their major polar lipids were diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine and their major fatty acids were $C_{16:1}\omega7c/C_{16:1}\omega6c$, $C_{16:0}$, $C_{18:1}\omega7c/C_{18:1}\omega6c$ and $C_{12:0}$. Both strains contained Q-8 as the only respiratory quinone component. The 16S rRNA gene sequences of the two strains possessed 99.1 % similarity; however, the level of DNA–DNA reassociation was only 26.7 %. The strains can also be discriminated from each other by several chemotaxonomic and biochemical traits. Phylogenetic analysis of the 16S rRNA gene sequences revealed the affiliation of both strains with the genus *Limnohabitans* within the family *Comamonadaceae*. The two investigated strains represent the first isolated members of a narrow phylogenetic cluster (the so- called R-BT065 cluster) formed by a large number of environmental sequences and abundant populations detected in the pelagic zones of various freshwater habitats. We propose to place the two strains in separate novel species within the genus *Limnohabitans*, *Limnohabitans planktonicus* sp. nov., with the type strain II-D5^T (=DSM 21594^T =CIP 109844^T), and Limnohabitans parvus sp. nov., with the type strain II-B4^T (=DSM 21592^T =CIP 109845^T). The description of the genus *Limnohabitans* is emended accordingly.

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Paper no. 3

Hahn MW, **Kasalický V,** Jezbera J, Brandt U & Šimek K (2010) *Limnohabitans australis* sp. nov., isolated from a freshwater pond, and emended description of the genus *Limnohabitans. Int J Syst Evol Microbiol* **60:** 2946 – 2950.

(IF = 1.930)

Abstract

A chemo-organotrophic, aerobic, non-motile strain, MWH-BRAZ-DAM2D^T, isolated from a freshwater pond in Brazil, was characterized phenotypically, phylogenetically and chemotaxonomically. Phylogenetic analysis of 16S rRNA gene sequences indicated affiliation of the strain with the genus *Limnohabitans* (*Comamonadaceae*, *Betaproteobacteria*). 16S rRNA gene sequence similarities between the isolate and *Limnohabitans curvus* MWH-C5^T, representing the type species of the genus, and the type strains of *Limnohabitans parvus* and *Limnohabitans planktonicus* were 98.2, 96.5 and 97.0 %, respectively. DNA–DNA reassociation analyses with DNA of the type strains of all three previously described *Limnohabitans* species revealed similarity values in the range 26.2–44.6 %. The predominant fatty acids of the isolate were $C_{16:1}\omega7c/\omega6c$, $C_{16:0}$, $C_{12:0}$ and $C_{8:0}$ 3-OH, the major quinone was ubiquinone Q-8 and the DNA G+C content was 55.8 mol%. The isolate could be discriminated from the type strains of the three *Limnohabitans* species by several phenotypic traits including differences in the utilization of several carbon sources. Based on the phylogeny of the isolate and its differences from the three most closely related species, the isolate represents a novel species for which the name *Limnohabitans australis* sp. nov. is proposed. The type strain is MWH-BRAZ-DAM2D^T (=DSM 21646^T =CCUG 56719^T).

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DOI: 10.1099/ijs.0.022384-0

Paper no. 4

Šimek K, **Kasalický V,** Jezbera J, Jezberová J, Hejzlar J & Hahn MW (2010) Broad habitat range of the phylogenetically narrow R-BT065 cluster representing a core group of the betaproteobacterial genus *Limnohabitans.* Appl Environ Microbiol **76:** 631-639.

(IF = 3.778)

Abstract

The distribution of the phylogenetically narrow R-BT065 cluster (*Betaproteobacteria*) in 102 freshwater lakes, reservoirs, and various ponds located in central Europe (a total of 122 samples) was examined by using a cluster-specific fluorescence in situ hybridization probe. These habitats differ markedly in pH, conductivity, trophic status, surface area, altitude, bedrock type, and other limnological characteristics. Despite the broad ecological diversity of the habitats investigated, the cluster was detected in 96.7% of the systems, and its occurrence was not restricted to a certain habitat type. However, the relative proportions of the cluster in the total bacterioplankton were significantly lower in humic and acidified lakes than in pH-neutral or alkaline habitats. On average, the cluster accounted for 9.4% of the total bacterioplankton (range, 0 to 29%). The relative abundance and absolute abundance of these bacteria were significantly and positively related to higher pH, conductivity, and the proportion of low-molecular-weight compounds in dissolved organic carbon (DOC) and negatively related to the total DOC and dissolved aromatic carbon contents. Together, these parameters explained 55.3% of the variability in the occurrence of the cluster. Surprisingly, no clear relationship of the R-BT065 bacteria to factors indicating the trophic status of habitats (i.e., different forms of phosphorus and chlorophyll a content) was found. Based on our results and previously published data, we concluded that the R-BT065 cluster represents a ubiquitous, highly active segment of bacterioplankton in nonacidic lakes and ponds and that alga-derived substrates likely form the main pool of substrates responsible for its high growth potential and broad distribution in freshwater habitats.

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Paper no. 5

Šimek K, **Kasalický V,** Horňák K, Hahn MW & Weinbauer MG (2010) Assessing niche separation in coexisting *Limnohabitans* strains through interactions with a competitor, viruses, and a bacterivore. *Appl Environ Microbiol* **76:** 1406–1416.
(IF = 3.778)

Abstract

We investigated potential niche separation in two closely related (99.1% 16S rRNA gene sequence similarity) syntopic bacterial strains affiliated with the R-BT065 cluster, which represents a subgroup of the genus *Limnohabitans*. The two strains, designated B4 and D5, were isolated concurrently from a freshwater reservoir. Differences between the strains were examined through monitoring interactions with a bacterial competitor, *Flectobacillus* sp. (FL), and virus- and predator-induced mortality. Batch-type cocultures, designated B4+FL and D5+FL, were initiated with a similar biomass ratio among the strains. The proportion of each cell type present in the cocultures was monitored based on clear differences in cell sizes. Following exponential growth for 28 h, the cocultures were amended by the addition of two different concentrations of live or heat-inactivated viruses concentrated from the reservoir. Half of virus-amended treatments were inoculated immediately with an axenic flagellate predator, *Poterioochromonas* sp. The presence of the predator, of live viruses, and of competition between the strains significantly affected their population dynamics in the experimentally manipulated treatments. While strains B4 and FL appeared vulnerable to environmental viruses, strain D5 did not. Predator-induced mortality had the greatest impact on FL, followed by that on D5 and then B4. The virus-vulnerable B4 strain had smaller cells and lower biomass yield, but it was less subject to grazing. In contrast, the seemingly virus-resistant D5, with slightly larger grazing-vulnerable cells, was competitive with FL. Overall, our data suggest contrasting ecophysiological capabilities and partial niche separation in two coexisting *Limnohabitans* strains.

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Paper no. 6

Šimek K, **Kasalický V,** Zapomělová E & Horňák K (2011) Algal-derived substrates select for distinct betaproteobacterial lineages and contribute to niche separation in *Limnohabitans* strains. *Appl Environ Microbiol* **77:** 7307–7315.

(IF = 3.778)

Abstract

We examined the proportions of major *Betaproteobacteria* subgroups within bacterial communities in diverse nonaxenic, monospecific cultures of algae or cyanobacteria: four species of cryptophyta (genera *Cryptomonas* and *Rhodomonas),* four species of chlorophyta (genera *Pediastrum, Staurastrum,* and *Chlamydomonas),* and two species of cyanobacteria (genera *Dolichospermum* and *Aphanizomenon).* In the cryptophyta cultures, *Betaproteobacteria* represented 48 to 71% of total bacteria, the genus *Limnohabitans* represented 18 to 26%, and the *Polynucleobacter* B subcluster represented 5 to 16%. In the taxonomically diverse chlorophyta group, the genus *Limnohabitans* accounted for 7 to 45% of total bacteria. In contrast, cyanobacterial cultures contained significantly lower proportions of the *Limnohabitans* bacteria (1 to 3% of the total) than the cryptophyta and chlorophyta cultures. Notably, largely absent in all of the cultures was *Polynucleobacter necessarius* (*Polynucleobacter* C subcluster). Subsequently, we examined the growth of *Limnohabitans* strains in the presence of different algae or their extracellular products (EPP). Two strains, affiliated with *Limnohabitans planktonicus* and *Limnohabitans parvus*, were separately inoculated into axenic cultures of three algal species growing in an inorganic medium: *Cryptomonas* sp., *Chlamydomonas noctigama,* and *Pediastrum boryanum.* The *Limnohabitans* strains cocultured with these algae or inoculated into their EPP consistently showed (i) pronounced population growth compared to the control without the algae or EPP and (ii) stronger growth stimulation of *L. planktonicus* than of *L. parvus.* Overall, growth responses of the *Limnohabitans* strains cultured with algae were highly species specific, which suggests a pronounced niche separation between two closely related *Limnohabitans* species likely mediated by different abilities to utilize the substrates produced by different algal species.

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Manuscript no. 7

Kasalický V, Jezbera J, Hahn MW & Šimek K Unveiling the diversity of the *Limnohabitans* genus, an important group of freshwater bacterioplankton, by characterization of 35 isolated strains. Submitted manuscript.

Unveiling the diversity of the *Limnohabitans* genus, an important group of freshwater bacterioplankton, by characterization of 35 isolated strains

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and IGS1 sequences of new *Limnohabitans* strains are HE600660-HE600692. The list of all sequences from bacterial strains and environmental clones used in this work is attached as supplementary material in Suppl. Mat. Table 3.

Running title: *Limnohabitans* genus microdiversity

Abbreviations: FISH - Fluorescence *In Situ* Hybridization, IGS1 - intergenic spacer between 16S and 23S rRNA genes

Keywords: freshwater, bacteria, Betaproteobacteria, Limnohabitans, microdiversity, morphology, isolated strains, FISH, R-BT065, 16S rRNA, IGS, ITS, metabolism, substrates

Abstract

Bacteria of the genus *Limnohabitans*, more precisely the RBT lineage, have a prominent role in freshwater bacterioplankton communities due to their high rates of substrate uptake and growth, growth on algal-derived substrates and high mortality rates from bacterivory. Moreover, due to their generally larger mean cell volume, compared to typical bacterioplankton cells, they contribute over-proportionally to total bacterioplankton biomass. Here we present genetic, morphological and ecophysiological properties of 35 bacterial strains affiliated with the *Limnohabitans* genus newly isolated from 11 non-acidic European freshwater habitats. The low genetic diversity indicated by the previous studies using the ribosomal SSU gene highly contrasted with the surprisingly rich morphologies and different patterns in substrate utilization of isolated strains. Therefore, the intergenic spacer between 16S and 23S rRNA genes was successfully tested as a fine-scale marker to delineate individual lineages and even genotypes. For further studies, we propose the division of the *Limnohabitans* genus into five lineages (provisionally named as LimA, LimB, LimC, LimD and LimH) and also additional sublineages within the most diversified lineage LimC. The microdiversity within the *Limnohabitans* genus should be taken into the careful consideration in ecological studies.

Introduction

Betaproteobacteria frequently belong to the most abundant members of freshwater bacterioplankton (Glöckner et al. 1999, Lindström et al. 2005). It is assumed that only four (Zwart et al. 2002) or seven (Newton et al. 2011) main tribes are present in freshwater habitats worldwide. The genus *Limnohabitans* (*Betaproteobacteria, Comamonadaceae*) has been recently established (Hahn et al. 2010a) as a group of environmentally important "not-easily cultivable" freshwater bacteria from the Beta-I lineage (Glöckner et al. 2000). The genus is currently composed of four described *Limnohabitans* species (Hahn et al. 2010a,b, Kasalický et al. 2010) and four lineages (Lhab-A1 to A4) that have been proposed within the genus (Newton et al. 2011). Two species, *L. planktonicus* and *L. parvus* (Kasalický et al. 2010), belong to the RBT lineage, targeted by the R-BT065 FISH (fluorescence *in situ* hybridization) probe (Šimek et al. 2001). Just recently, a large database containing environmental sequences from RBT group has been established (Newton et al. 2011).

The bacteria from the RBT lineage are known to inhabit a broad range of freshwater habitats within at least three continents and can constitute up to 30 % of free-living bacteria in freshwater systems (Glöckner et al. 2000, Zwart et al. 2002, Page et al. 2004, Šimek et al. 2010b). It has been shown that they strongly prefer non-acidic habitats and their abundance in low pH habitats is usually negligible (Šimek et al. 2010b). In lakes, they inhabit the neuston (Hörtnagl et al. 2010), the epilimnion (e.g. Šimek et al. 2001), and the hypolimnion (Buck et al. 2009, Salcher et al. 2010), indicating their capabilities to live in both oxic and anoxic environments (Buck et al. 2009, cf. Kasalický et al. 2010).

The RBT lineage is known to be represented by phylotypes with opportunistic strategies (Šimek et al. 2005, Salcher et al. 2007). The RBTs are characterized by a high percentage of cells incorporating leucine (Horňák et al. 2006, Salcher et al. 2008, Buck et al. 2009, Pérez et al. 2010) and glucose (Buck et al. 2009), whereas low uptake rates were measured for thymidine (Horňák et al. 2006, Pérez et al. 2010) and acetate (Buck et al. 2009) and no uptake for the incorporation of 4-hydroxybenzoic acid (Buck et al. 2009). Notably, the RBT bacteria displayed the highest growth rate among major

bacterioplankton lineages, comparable to growth rates of small heterotrophic nanoflagellates under *in situ* conditions (Šimek et al. 2006). Interestingly, experimentally manipulated grazing pressure markedly accelerated growth of R-BT065 targeted bacteria (Šimek et al. 2007), which were moreover preferentially ingested by these flagellates (Jezbera et al. 2006). Further, these results were complemented with a specific study examining niche separation in two closely related species of *L. parvus* and *L. planktonicus* (Šimek et al. 2010a), based on their size, growth capabilities, vulnerability to protozoan grazing, and virus infection.

The predominant natural source of substrates for the RBTs seems to be autochthonous algal-derived organic material (Pérez & Sommaruga 2006, Šimek et al. 2008, 2010b). Notably, growth of *L. parvus* and *L. planktonicus* on algal exudates as a sole dissolved organic carbon (DOC) source has just been confirmed (Šimek et al. in 2011). Products of the photolysis of dissolved organic matter have also been suggested as an important additional source of substrates for these bacteria (Glaeser et al. 2010, Hörtnagl et al. 2010).

In contrast to the considerable information on the ecophysiology of the RBT group, we have almost no knowledge on the ecology of the other two described *Limnohabitans* species, *L. curvus* and *L. australis* (Hahn et al. 2010a,b), since no specific FISH probe is currently available to follow their *in situ* population dynamics.

The extremely wide range of habitats (4.9 - 9.1 pH, Šimek et al. 2010b) occupied in combination with the marked ecophysiological capabilities of RBT bacteria (see above), suggest a large microdiversity within the cluster. However, existing 16S rRNA gene sequences show more than 96% identity, suggesting either that genetic diversity is low or that 16SrRNA is inappropriate for diversity assessment. To distinguish between these two possibilities, we established comprehensive sets of molecular and ecological data in a polyphasic approach building on additional representative strains isolated from the *Limnohabitans* genus and the RBT lineage.

In this paper, we characterize ecophysiological patterns and analyze the phylogeny and morphology of 35 newly isolated strains affiliated within the *Limnohabitans* genus. The aims of the presented study were: (i) to examine the

diversity within the *Limnohabitans* genus by sequencing of the 16S rRNA gene and the IGS1 loci of the newly isolated *Limnohabitans* strains and characterization of phylogenetically distinct lineages within the genus, (ii) to investigate metabolic capabilities and morphological and size-related characteristics of the isolated strains and to interpret these phenotypic traits regarding potential differences in ecological adaptations, and (iii) to reassess the contribution of RBT bacteria to total abundance and biomass of bacterioplankton in seven ecologically contrasting habitats.

Results

Growth abilities and morphological traits of isolated strains

Thirty-five bacterial strains affiliated within the *Limnohabitans* genus were isolated from 12 ecologically diverse freshwater habitats (Table 1). Seven habitats can be assigned to the category “Fishponds and reservoirs”, four to “Alkaline lakes”, one to “Small shallow ponds” as predefined in Šimek et al. (2010b). However, we failed to isolate *Limnohabitans* strains from low pH habitats such as "Humic lakes and ponds" or "Acidified lakes".

Usually, one or two RBT-positive wells were present among 100 to 150 wells displaying turbidity, however the proportion of *Betaproteobacteria*-positive wells was always much higher and varied broadly. Interestingly, this ratio was several-fold higher for the Klíčava compared to the Římov reservoir. Therefore, we obtained with smaller effort almost twice as many isolates from the Klíčava reservoir.

All isolated strains were screened microscopically for their shape and size at the end of the acclimation procedure and during the purification, and regularly checked by FISH with the R-BT065 and the Bet42a probes. The isolated strain morphologies were: coccoid, ovoid or short-rod (20 strains), rod (1 strain), curved rod (2 strains), solenoid (8 strains) or large solenoid/C-shaped morphology (5 strains, see Fig. 1). Cell sizes spanned over a wide range of sizes from 0.4 µm-diameter of cocci up to 5 µm in length of curved rods (for details see Table 2).

Genetic diversity

Almost complete sequences of 16S rRNA (1435–1440 bp) genes and complete sequences of IGS1 regions (648–771 bp, including 2 tRNAs – Ile and Ala) were obtained for all isolated strains. In addition, complete IGS1 sequences were obtained for *L. curvus* MWH-C5^T, *L. australis* MWH-BRAZ-DAM2D^T, *L. parvus* II-B4^T, *L. planktonicus* II-D5^T, *Rhodoferax fermentans* FR2^T and *Curvibacter gracilis* 7-1^T.

Phylogenetic analysis of the 16S rRNA gene sequences, including validly described species and environmental samples, supported the affiliation of the isolated strains within the genus *Limnohabitans* (Fig. 2). All strains also possess the target sequence for the Rho-BAL47 probe (Zwart et al. 2002). Five main lineages (provisionally named LimA, LimB, LimC, LimD and LimE) were consistently observed in phylogenetic trees constructed using different algorithms (NJ, MP, ML, bayesian) confirming the robustness of the phylogenetic grouping within the genus *Limnohabitans*.

Lineage LimA (identical to Lhab-A3 in Newton et al. 2011) is the only group within the genus which does not possess the discriminative sequence 5'- GTT GCC CCC TCT ACC GTT -3' matching the R-BT065 probe, and consequently their members remain "invisible" by using this probe. Two already described species, *L. curvus* and *L. australis*, (Hahn et al. 2010a, b) and 5 newly isolated strains are affiliated within this lineage. All 7 strains are morphologically similar, of a solenoid shape (Fig 1A, 1B and Hahn et al. 2010a,b). The 5 new members were isolated from 4 different habitats and they clustered together with other related cultivated strains and environmental sequences available in GenBank a well-separated lineage within the *Limnohabitans* genus. The similarity within the lineage is > 98 % on 16S rRNA gene and > 89 % on IGS1 sequence. The length of the IGS1 sequence is 612–622 bp. The new strains KL6S and Rim8 isolated from different habitats (Tables 1 and 2), shared both sequences identical with strain *L. curvus* MWH-C5^T, thus they most probably represent the same species. All the phylogenetic algorithms used suggested a separation of the strain *L. australis* MWH-BRAZ-DAM2D^T vis-a-vis other isolated strains and environmental sequences.

Phylogenetic analyses of both 16S rRNA and IGS1 genes of isolated strains indicate that the large Lhab-A1 group (Newton et al. 2011) is consistently separated into two sister lineages. We would like to stress this delineation to minimize the number of potential genotypes present in each taxonomic unit, and we propose to call the lineages LimC and LimB. **Lineage LimB** is represented by three newly introduced strains (Fig 1B, C, Table 2) and also contains environmental sequences originating from different aquatic habitats in Switzerland, Austria, Germany, China and 7 states in the USA. The strains within the lineage share similarities of their 16S rRNA gene > 99.5 % and of their IGS1 sequence > 89.9 %. The new strains were isolated from the Klíčava and Římov reservoirs. Their cells are rather small, cocci to short rods, with the volume 0.03–0.05 μm^3. The existence of the LimB lineage has been previously indicated by clone PRD01b009B (AF289169) and related sequences from Lake Michigan, where it formed the highest proportion of clones of freshwater *Betaproteobacteria* (Mueller-Spitz et al. 2009).

Lineage LimC includes two described species *L. planktonicus* and *L. parvus*, 25 newly isolated strains presented in this study (Table 2) and other environmental sequences. The origin of the sequences affiliated within the LimC lineage is worldwide (e.g. Europe, USA, Argentina, Taiwan and China) including not only freshwaters and estuaries but also non-freshwater habitats as epithelium of *Hydra vulgaris* (Fraune & Bosch 2007) and digestive tract of *Daphnia magna* (Freese & Schink 2011). This lineage harbors all the bacterial morphotypes found, i.e. cocci, rods and solenoid bacteria (cf Fig 1). The affiliated strains share similarities in both their 16S rRNA gene (> 98.4%) and of their IGS1 sequence (> 89%). The length of the IGS1 sequence is 712–746 bp. To better understand their ecology, we propose the following annotation and differentiation, as it is indicated by morphologically identical genetic clusters (Table 2, Fig 2, 3). **LimC1** and **LimC2 sublineages** are proposed for species clusters of *L. planktonicus* and *L. parvus* respectively. The morphological and genetic similarities of strains 2KL-16 (Fig 1J) and Rim42 with *L. planktonicus* II-D5^T suggest that they probably represent the same species. Strains LI2-55 (Fig 1H) and VIII-A6 possess identical IGS1 and 16S rRNA gene sequences as strain *L. parvus* II-B4^T and similar morphology, thus they likely represent the same species. However, strain LI2-

55 was isolated from a habitat located 700 km far from the habitat of VIII-A6 and II-B4^{T}. The **sublineage LimC3** harbors two coccoid strains CEP5 (Fig 1F) and T6-20 isolated from habitats with high nutrient concentration. The **sublineage LimC4** is proposed for strains B22-3k and Hin4 (Fig 1I), representing short rods/cocci. The strains were recovered from two different types of habitat, an eutrophic pond and a calcareous alpine lake. The morphologically exceptional **sublineage LimC6** (cf. Fig 1L) is composed of strains 2KL-3, G3-3, SP2 and WS1 which are characterized by largest MCV (up to 1 µm^{3}) found within *Limnohabitans* spp. so far, as well as by a clearly distinguishable C-shaped morphology. All four strains belong to one genotype as both 16S rRNA gene and IGS1 sequences are identical. The length of their IGS1 sequence is 736 bp.

The existence of **LimD lineage** is highly supported by bootstrap analysis (Fig. 3), however, it still does not include any isolated strain and is defined exclusively on the basis of the corresponding environmental sequences obtained from Genbank. This group has been previously associated within Lhab-A2 tribe (Newton et al. 2011) with other phylogenetically unrelated clones (Newton's ARB database), which could lead to data misinterpretation. Therefore, we propose the clones FukuN55 (AJ289999) and PIB-25 (AM849436) as "type sequences" for this lineage. The sequences clustering within this lineage originate from oligo- to mesotrophic lakes in Austria, Germany and Switzerland (Glöckner et al. 2000, Crump et al. 2003, Percent et al. 2008, Salcher et al. 2008) as well as from estuary of Delaware river (Shaw et al. 2008).

LimE lineage consists only of two strains isolated from the same habitat, however, morphologically distinct (Table 2). Its members are genetically close to lineage LimA, but they can be hybridized with R-BT065 probe (cf Fig 2 and Fig 3). They share similarities in both their 16S rRNA gene (> 98.4%) and of their IGS1 sequence (> 89%). The length of the IGS1 sequence is 615–723 bp. This lineage could probably include the "R-BT065" group indicated in Newton's ARB database, represented by 58 clones exclusively from the Lake Michigan (Mueller-Spitz et al. 2009), e.g. clones LW1m-1-53 (EU639913) and GC1m-1-33 (EU641261).

Biovolume of RBTs

Volumes of all heterotrophic and all R-BT065 probe-positive bacteria were determined for 7 different habitats and selected on the basis of our previous knowledge on RBT bacteria abundance (Fig. 4). Volume of RBT-positive bacteria ranged from 0.003 to 0.224 μm^3 whereas the volume of non-RBT bacteria ranged from 0.003 to 0.685 μm^3 for all habitats. In all examined habitats, R-BT065 probe targeted bacteria cells on average larger than non-RBT cells ($p<0.001$, Fig. 4A). The relative contribution of RBTs to total bacterial biomass in the cellular carbon was in all cases higher than their relative abundance ($Z=2.366$, $p=0.016$, Fig. 4B).

Discussion

Betaproteobacteria – ecological relevance versus available isolates

One of the fundamental goals of the field freshwater microbial ecology is connecting our rather limited knowledge on the "not-easily cultivable" but key bacterioplankton taxa with their major environmental functions (Newton et al. 2011). Due to the inherent difficulty in the cultivability of aquatic bacteria (e.g. Zwart et al. 2002, Hahn et al. 2004), the mosaic of the relevant taxonomic units and especially their function remains largely incomplete. In this study, we present a first overview of the morphological, genetic and physiological microdiversity within the *Limnohabitans* genus based on newly isolated strains with a large potential to link data on genetic diversity to data on phenotypic diversity and ecological roles of particular taxonomic units.

Freshwater *Betaproteobacteria* represent a group of heterotrophic bacteria with the largest number of so far isolated strains, although most of them belong to the *Polynucleobacter* genus (Hahn et al. 2005, Hahn et al. 2009, Wu & Hahn 2006, Hahn et al. 2012). Our study reports on 35 newly isolated strains from the *Limnohabitans* genus (Hahn et al. 2010a) an important unit of the betaI tribe (Zwart et al. 2002). Notably, another 16 *Limnohabitans* strains were recently isolated from lakes Teganuma, Inbanuma, Inawashiro and Ushikunuma on Japan islands (K. Watanabe et al., unpublished results). Thus, including four described species there are currently at least 55 strains available for further studies.

Revision of the phylogenetic scheme for betaI tribe

Hundreds of partial 16S rRNA gene sequences in Genbank (www.ncbi.nih.gov) retrieved by cultivation-independent approaches and affiliated within the RBT lineage and/or the genus *Limnohabitans* give the potential of a plausible phylogenetic reconstruction of the genus (Kasalický et al. 2010, Newton et al. 2011, Fig 2 in this study). Our newly isolated strains form a monophyletic cluster within the *Limnohabitans* genus with high similarities of their 16S rRNA gene sequences (Suppl. Mat. Table 1). Phylogenetic analysis of 16S rRNA genes revealed five main lineages

within the genus (Fig. 2), which enlarges the number of tribes proposed by Newton and co-workers (2011). However, some of our phylogenetic reconstructions contradict the proposals presented in their review paper.

Newton and co-workers synonymized GKS16 cluster, proposed by Zwart et al. (2002), with lineage Lhab-A2 and placed this taxon into the *Limnohabitans* genus (Fig. 6B in Newton et al. 2011). In addition, they indicated that GKS16/Lhab-A2 is targeted by the R-BT065 probe. However, our phylogenetic analyses do not support such a grouping. Lhab-A2 labeled sequences in the Newton's ARB database are paraphyletic with two independent lineages: the GKS16 cluster represented by clones GKS16 (AJ224987) or JEG.e1 (DQ228403) and the lineage LimD (Suppl Table 3). According to the results of Zwart and co-workers and results presented in this work, the GKS16 cluster represents an independent lineage closely related to the *Polaromonas* genus (Fig 2). Moreover, all GKS16 affiliated clones lack the discriminatory sequences targeted by the R-BT065 or Rho-BAL47 probe which highly contrasts with the clones affiliated within the LimD lineage. Ecological data clearly support the phylogeny: the sequences affiliated within the GKS16 lineage were retrieved almost exclusively from cold habitats (i.e. snow, ice core, arctic streams), whereas no *Limnohabitans* sequences were obtained from such habitats to date. However, there is some evidence that both lineages can co-occur in the same habitat, e.g. high mountain lakes (Pérez and Sommaruga 2006 and Šimek et al. 2010b).

The affiliation of the lineage Lhab-A4 within the *Limnohabitans* genus is highly questionable. The phylogenetic analyses suggest the position of Lhab-A4 as a sister lineage to the *Limnohabitans* genus or at the edge of this genus. Moreover, none of the Lhab-A4 clones, e.g. clones ADK-MOe02-95 (EF520475) and LW9m-3-24 (EU641662), contain the target sequence for the R-BT065 probe, however they could be targeted with Rho-BAL47 probe. Nevertheless, the lack of isolated members does not allow to resolve whether Lhab-A4 lineage could be assigned to the *Limnohabitans* genus or not.

Thus in contrast to the previously proposed phylogenetic scheme (Newton et al. 2011), there is compelling evidence for the existence of five lineages of the

Limnohabitans genus: four lineages representing the RBT bacteria and one lineage (LimA) for non-RBT bacteria (Fig 2 and 3). Based on the resolution of our phylogenetic analysis on existing isolated strains, we propose a new phylogenetic scheme for the betI group and new names for the respective lineages within the *Limnohabitans* genus (Fig. 2), which substantially refines and clarifies the scheme suggested by Newton et al. (2011).

Fine-scale resolution within the genus

The availability of a broad spectrum of strains from the same lineage allows testing the suitability of markers for a finer resolution at the species-level in natural habitats. An important contribution of our research is the sequencing of the highly variable 16-23S rRNA intergenic spacer (IGS1). To the best of our knowledge, IGS1 sequences of uncultured or cultivated *Limnohabitans* strains were not previously deposited in Genbank. An explanation of the widespread avoidance of IGS1 sequencing is the possible presence of multiple operons of the ribosomal genes and the presence of the multiple non-identical IGS1 sequences in a single genome (Boyer et al. 2001). However, only two rRNA operons, but with identical IGS1 sequences, were reported in closely related *Rhodoferax ferrireducens* genome (Risso et al. 2009), and only one rRNA operon seems to be present in a common freshwater betaproteobacterium *Polynucleobacter necessarius* spp. *asymbioticus* genome (Hahn et al. 2012). Moreover, the highest intragenomic divergence of IGS1 sequences within *Betaproteobacteria* was reported being about 5 % (Stewart & Cavanaugh 2007), while we found a IGS1 sequence similarity higher than 89 % within proposed lineages (Suppl. Mat. Table 2), thus in concordance with 16S rRNA affiliation. On the other hand, neither IGS1 sequences supported the definition of larger phylogenetic clusters. Therefore, we decided not to insist on the description of additional new sublineages. For this purpose other gene(s) should be sequenced.

IGS1 sequences have been frequently used to distinguish closely related strains (Hahn et al. 2005, Wu et al. 2010, Hofmann et al. 2010, Jezbera et al. 2011). Therefore, five genotype groups (E, F, G, *curvus* and *parvus*), including two to four strains with similar size and shape and identical IGS1 and 16S rRNA gene sequences and isolated

from more than one habitat, were explicitly proposed as new well-defined taxonomic units (cf. Fig. 2 and 3). Regarding the morphological features of the isolated strains, we hypothesize that the lower limit of the IGS1 similarity within an individual genotype is about 95 % (Suppl. Mat. Table 2), which permits consideration of all other strains as genotypes as-well. However, the similarity of genes and the similarity of the whole bacterial genomes do not correlate (Stackebrandt & Ebers 2006), thus additional genetic and physiologic tests are needed to verify our hypothesis since further splitting of the proposed sublieneages (or groups) could not be ruled out.

Contrary to our expectations, it seems impossible to draw firm conclusions on habitat preferences of proposed *Limnohabitans* (sub-) lineages based solely on 16S rRNA sequences deposited in Genbank (Suppl. Mat. Fig 1). Apparently, there are three reasons: (i) too low phylogenetic and the so far underestimated diversity of this group huge microdiversity, (iii) only limited knowledge on the ecology of this group of bacteria. Also in this case, the ecological diversification of freshwater bacteria is undoubtedly deeper than currently mirrored by available molecular data (cf. Jezbera et al. 2011).

Are there common traits among* Limnohabitans *members?

The members of the RBT lineage are found in a large variety of mostly freshwater habitats (e.g. Salcher et al. 2007 and 2008, Buck et al. 2009, cf. Newton et al. 2011). In these systems, they are among the most metabolically active groups and are subjected to high grazing-induced mortality (Šimek et al. 2005, 2007, Jezbera et al. 2005, Salcher et al. 2008). The ability to respond to changing conditions, called "metabolic IQ" (Galperin 2005), has been suggested to be correlated with the bacterial genome size and in turn also with their cell volume (Yooseph et al. 2010). If these assumptions are correct, the generally larger cell volume and the growth potential of the RBT bacteria (Fig 4) indicate that they belong to the opportunistic (i.e. more substrate-responsive) fraction of the bacterioplankton (cf. Salcher et al. 2008), thus this "substrate versatility" seems to be a common trait shared within RBT bacteria.

Environmental factors such as pH, conductivity, and the proportion of low-molecular-weight compounds in dissolved organic carbon were found to correlate with

their abundance (Šimek et al. 2010b). The genetic libraries contain only a few betaI clones obtained from acidic habitats, i.e. Adirondack Lakes (Percent et al. 2008). However, all clones affiliated to the *Limnohabitans* genus were indigenous to the lakes with pH around 7, e.g. ADK-CSe02-53 (EF520468) from Cascade Lake. Thus, the low pH seems to severely limit most of *Limnohabitans* bacteria. However, one might expect that important exceptions would be uncovered by future detailed studies of the bacterial community composition of acidic or acidified lakes. Surprisingly, no significant correlation of the abundance of RBT bacteria with lake trophic status and chlorophyll concentration was found (Šimek et al. 2010b), nor any clear habitat preference can be determined for individual lineages from the phylogenetic distribution of sequences deposited in Genbank (Suppl. Mat. Table 3).

Large potential for ecological differentiation

The success in isolation of strains possessing different genotypes from the same habitat or even from the same water sample (Table 1 and 2) and the existence of clone libraries with sequences distributed throughout all *Limnohabitans* lineages (e.g. Shaw et al. 2008) suggest that their coexistence is likely facilitated by their different ecophysiological traits. In addition, the high abundance of *Limnohabitans* members (in average $0.3 \cdot 10^6$ ml^{-1}, Šimek et al. 2010b) together with large genetic diversity (cf. DNA-DNA hybridization values in Hahn et al. 2010a,b and Kasalický et al. 2010) indicate a huge potential for diversification and speciation.

Three putative mechanisms for the speciation and niche differentiation within the same body of water can be proposed based on physiological traits of isolated strains and available knowledge on the RBT lineage ecology.

Metabolic capabilities of the selected strains (Table 3) are assumed to give them a specific physiological potential to exploit available organic carbon. The quality of the organic matter is not only coupled to its allochthonous and autochthonous origin (e.g. Pérez & Sommaruga 2006), but also to individual algal or cyanobacterial producers (e.g. Giroldo & Vieira 2002, 2005). The changes in bacterial community composition, and species-specific algal-bacterial relationships have been documented in both marine and freshwaters (Grossart et al. 2005, Horňák et al. 2008, Alonso et al.

2009). Moreover, the algal-specific coupling was described for RBT bacteria (Horňák et al. 2008, Šimek et al. 2008, Šimek et al. 2011). The investigations on the potential between two tested *Limnohabitans* species to use algal derived organic matter showed significant differences in their growth characteristics (Šimek et al. 2011).

The morphological and size-related diversity present within the RBT cluster (see Figs. 1 & 4) likely corresponds with a diversity in vulnerability to grazing. This is supported by investigations into the ecological traits of two closely related, but in size and morphology rather dissimilar bacteria, i.e. *L. planktonicus* and *L. parvus* (Šimek et al. 2010a). Strain-specific differences in the vulnerability to flagellate grazing and to viral infection (Šimek et al. 2010a) suggest that these species occupy separated ecological niches (cf. Boenigk et al. 2004). The cell volume of the newly isolated strains encompass a range from 0.03 up to 1 μm^3 (Table 2), thus according to marine bacteria their genome size could range from about 1.6 to 6 Mbp (cf. Yooseph et al. 2010). Although these approximations might be incorrect, there is a certain possibility that at least some RBT bacteria could harbor small-sized genotypes with a low metabolic potential. Then for escaping grazing pressure they could exploit the so-called "cryptic escape" lifestyle suggested by Yooseph et al. (2011) instead of the above mentioned opportunist strategy.

Finally, the presence of members of the *Limnohabitans* genus have been reported by non-cultivable methods from atypical aquatic sites: the epithelium of free-living *Hydra* (Fraune & Bosch 2007), and the gut microflora of *Daphnia magna* (Freese & Schink 2011, cf. Fig 2). It seems that such a possible symbiosis or mutualism might be more common for distinct aquatic bacterial genera. Similar types of associations were described for the freshwater genus *Polynucleobacter* (Vannini et al. 2007) or the marine genus *Vibrio* (Urbanczyk et al. 2007). These associations are highly (strain) specific and the bacterial symbiont occupies a privileged niche, which highly modifies its life strategy in an aquatic habitat.

Concluding remarks

Previously an uncultured bacterial group is now known to contain a large number of distinct members. We can assume that there is enough information to open a

black box frequently used in the research on freshwater microbial ecology (for review see Newton et al. 2011) and assign the target group of bacteria to new phylogenetically defined taxa with distinct phenotypic and ecological features. To determine the well-defined ecological units of the *Limnohabitans* genus, it is of the primary interest to study the biological interactions on the species- or even strain-level. In addition, there is an urgent need to establish narrower, high taxonomic-resolution markers to describe the occurrence, habitat preferences and ecological roles of individual *Limnohabitans* lineages and genotypes. We propose the IGS1 sequence as a more appropriate marker than the commonly used 16S rRNA gene for fine-scale phylogenetic studies within the *Limnohabitans* genus, and we provide a basic sequence dataset and a taxonomic framework both suitable for interpretation of clone libraries established by cultivation-independent methods.

Experimental Procedures

Isolation and identification of bacteria

Bacterial strains were isolated from freshwater reservoirs, lakes and ponds in the Czech Republic, Austria, the Netherlands and France (Corse) using a modified protocol of the acclimatization method (Hahn et al. 2004); for more details of the habitats used for isolation, see Table 1 and Šimek et al. (2010a). Two manipulation approaches were used to enrich bacteria affiliated with the *Limnohabitans* genus, either a grazer removal or a sample dilution approach. The first protocol, as described by Kasalický et al. (2010), employed the filtration of the whole water sample through 0.8 µm polycarbonate membrane filter (OSMONICS, Livermore, USA) to remove protists. In the second method, the whole water sample was diluted 1:1 with Inorganic Basal Medium (IBM, Hahn et al. 2004). After both manipulations, the samples were kept for 24 hours in dark, facilitating enhanced bacterial growth and activation, and subsequently diluted with sterile IBM medium in order to obtain cell concentrations suitable for inoculation of 24-well microplates with approximately 0.5 cells per well. Usually 6 to 10 microplates were used for one water sample. The established cultures were stepwise acclimatized by additions of increasing doses of NSY medium to growth at 3 g l^{-1} (Hahn et al. 2004). Wells showing turbidity were screened by FISH with the Bet42a (whole *Betaproteobacteria*, Manz et al. 1992) and the R-BT065 (RBT lineage, Šimek et al. 2001) probes for presence of *Limnohabitans* spp. Samples were scored as "positive" when the cells hybridized with the R-BT065 probe or solenoids hybridized only with Bet42a probe (for the strains related to *L. curvus* and *L. australis* (cf Hahn et al. 2010ab)). 500 µl from the positive wells were re-inoculated to fresh NSY medium and at least 3 times purified by dilution to extinction. The purity of cultures was controlled microscopically by DAPI staining, by FISH (e.g. Šimek et al. 2001), and by growth on agar plates (NSY medium). However, not all cultures were able to grow on solid media (1.5% agar), thus the latter test provided only partial or additional information on the purity of a culture based on colony size, shape and color.

Metabolic tests

The isolated strains were routinely grown in liquid NSY medium with strength of 3 g l^{-1}. Assimilation experiments were performed by comparison of optical density measured at 575 nm (OD_{575}) established in liquid one-tenth-strength NSY medium (0.3 g l^{-1}) with and without 0.5 g of a test substance per liter (pH 7.2), as described previously (Hahn et al. 2009). Differences in OD_{575} of 30% , 30–80% and more than 80% of the OD_{575} established on medium without test substance were scored as no assimilation, weak assimilation and assimilation, respectively.

Phylogenetic analysis

DNA from the established purified cultures was extracted by using the UltraClean™ isolation kit (MoBio, Laboratories, Inc.). The 16S rRNA genes and the intergenic spacer region between 16S and 23S rRNA genes (IGS1) were amplified using primers 27F, 1492r (both Weisburg et al. 1991), and 1406F (Lane et al. 1985), 23Sr (Fisher & Triplett 1999) as described in Hahn et al. (2005). The PCR products were purified by Nucleospin™ (MoBio, Laboratories, Inc.) Sequencing was performed commercially by Eurofins MWG Operon (Germany). To obtain IGS1 sequences of closely-related reference species, the following strains were grown in 3 g.l^{-1} NSY medium: *L. australis* MWH-BRAZ-DAM2D^T, *L. curvus* MWH-C5^T, *L. parvus* II-B4^T, *L. planktonicus* II-D5^T, *Curvus gracilis* 7-1^T and *Rhodoferax fermentans* FR2^T.

Sequences were aligned with MAFFT 6 (http://mafft.cbrc.jp/alignment/server, Katoh et al. 2002, Katoh & Toh 2008). Aligned sequences were edited in BioEdit 7.0 (Hall 1999). Similarities of aligned sequences were calculated by the Sequence Identity Matrix program in BioEdit 7.0, and pairwise distances were calculated with MEGA 5 (Tamura et al. 2011). Best model for Maximum Likelihood (GTR+Γ+I) analysis was estimated by jModelTest (Posada 2008). Neighbor-joining trees and Maximum Parsimony were calculated by using the software MEGA 5 (Tamura et al. 2011), Maximum Likelihood trees were generated using PhyML 3.0 (Guindon & Gascuel 2003), Bayesian evolution was calculated by using MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001).

Biovolume of the FISH-positive bacteria in natural samples

Natural samples (10 to 20 ml) for catalyzed reporter deposition FISH were sampled as described in Šimek et al. (2010b). Cells were hybridized with the R-BT065 oligonucleotide probe (Šimek et al. 2001). The proportions of FISH-positive bacteria were determined directly by inspecting 600 to 1,000 cells in replicated samples using epifluorescence microscopy (Olympus AX-70). Gray-scale images of bacterial cells were acquired with a CCD camera in two channels with distinct combination of excitation and emission light spectra. The "probe" channel was used to assign the R-BT065-positive cells to their image in "DAPI" channel. Cell sizing, based on measuring of cell width and length, was conducted in "DAPI" channel by using the semiautomatic image analysis system LUCIA D (Lucia 3.52; Laboratory Imaging, Prague, Czech Republic) as described by Posch et al. (1997 and 2009). Between 30 and 100 hybridized cells were measured per sample to determine the mean cell volume (MCV) of the R-BT065-positive bacteria. Cell volumes of probe detected and not-targeted bacteria were compared by Mann-Whitney U statistical test, since the normality distribution test failed ($p<0.001$).

Carbon content of individual cells was calculated according to Loferer-Krössbacher et al. (1998). The relative proportions of abundance and carbon biomass of R-BT065-positive cells in selected habitats were calculated using the cluster-specific abundance given in Šimek et al. (2010b) and were compared to the values for all bacterioplankton cells by Wilcoxon Signed Rank Test (pair t-test for data where normality test failed, $p=0.020$).

Nucleotide Sequences

16S rRNA gene sequences and 16S-23S IGS1 sequences of the *Limnohabitans* isolates and several reference strains were deposited under the Accession Numbers HE600660-HE600692. A detailed list of strains and the corresponding accession numbers is available in Suppl. Mat. Table 3.

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Habitat	No. of isolates	Habitat characteristics	Surface area (ha)	Geographic coordinates	Country	Trophic status	pH	Conductivity ($\mu S\ cm^{-1}$)
Klíčava reservoir	12	Reservoir for drinking water supply	74,1	50°3'58"N, 13°55'55"E	Czech Republic	mesoeutrophic	8,9	452
Římov reservoir	8	Reservoir for drinking water supply	206	48°50'N, 14°29'E	Czech Republic	mesoeutrophic	7 – 8	100 – 120
Bagr pond	3	shallow urban pond with a concrete bottom	1	48°58'17"N, 14°27'23"E	Czech Republic	eutrophic	7 – 8	300 – 400
Gosau 3	2	small shallow montane lake	0,04	47°35'23"N, 13°34'13"E	Austria	oligomesotrophic	7,5	493
Lake Loosdrecht	2	Large shallow peat lake	980	52°12'15.91"N, 5° 4'52.58"E	Netherlands	eutrophic	8,0	420
Lužnice pond T6	2	Small deep pond in Lužnice riverbed	0,01	48°50'0.453"N, 14°55'40.324"E	Czech Republic	eutrophic	6,8	200
Seepromenade	2	Small shallow urban pond in Mondsee	0,02	47°51'06,36"N, 13°21'04,34"E	Austria	eutrophic	7,4	341
Hintersee	1	Deep submontane lake in prealpine region	82	47°44'49"N, 13°14'59"E	Austria	oligomesotrophic	8,5	249
Mondsee	1	Deep submontane lake in prealpine region	1378	47°50'N, 13°20'E	Austria	oligomesotrophic	8,3	323
Nový u Cepu pond	1	shallow fishpond	10,5	48°55'16.912"N, 14°49'52.806"E	Czech Republic	hypertrophic	7	211
Wiestalstausee	1	Reservoir in prealpine region	70	47°44'47,46"N 13°10'13,96"E	Austria	oligomesotrophic	8,3	275

Table 1
Characteristics of freshwater habitats from which *Limnohabitans* spp. strains were isolated.

Isolate	Habitat	Lineage/ sublineage	Morphology		
			Cell length (μm)	Cell volume ($μm^3$)	Shape
B9-3	Bagr pond	LimE	0.5 – 1.2	0.04 – 0.11	ovoid
B10-3v	Bagr pond	LimE	0.5 – 1.1	0.03 – 0.06	solenoid
B22-3vk	Bagr pond	LimC4	0.6 – 1.0	0.05 – 0.10	short rods
G3-2	Gosau 3	LimC	0.4 – 0.7	0.04 – 0.07	coccoid
G3-3	Gosau 3	LimC6	2.1 – 3.0	0.30 – 0.52	large solenoid
Hin4	Hintersee	LimC4	0.4 – 0.6	0.02 – 0.04	short rods
KL1	Klíčava res.	LimB	0.5 – 1	nd	short rods
KL5	Klíčava res.	LimC5	0.7 -1.0	0.05 – 0.13	ovoid
KL6	Klíčava res.	LimC	0.5 – 0.8	0.04 – 0.07	short rods
KL6S	Klíčava res.	LimA	0.8 – 1.1	0.06 – 0.18	solenoid
2KL-1	Klíčava res.	LimC	0.8 – 1.4	0.08 – 0.21	solenoid
2KL-3	Klíčava res.	LimC6	2.3 – 3.4	0.41 – 0.78	large solenoid
2KL-5	Klíčava res.	LimC	0.5 – 1.0	0.04 – 0.13	short rods
2KL-7	Klíčava res.	LimC6	2.7 – 3.9	0.35 – 0.83	large solenoid
2KL-15	Klíčava res.	LimB	0.4 – 0.6	0.02 – 0.04	coccoid
2KL-16	Klíčava res.	LimC1	1.4 – 2.2	0.12 – 0.19	curved rods
2KL-17	Klíčava res.	LimC4	0.4 – 0.7	0.04 – 0.06	coccoid
2KL-27	Klíčava res.	LimC5	0.5 – 0.7	0.04 – 0.07	coccoid
LF5-52	Lake Loosdrecht	LimA	0.6 – 0.9	0.05 – 0.09	solenoid
LJ2-35	Lake Loosdrecht	LimC2	0.4 – 0.6	0.04 – 0.06	coccoid
Mo2-6	Lake Mondsee	LimC	0.3 – 0.6	0.02 – 0.04	coccoid
T6-5	Lužnice pond T6	LimC	1.7 – 3.1	0.31 – 0.90	curved rods
T6-20	Lužnice pond T6	LimC3	0.6 – 0.9	0.04 – 0.07	coccoid
CEP5	Nový u Cepu fishpond	LimC3	0.4 – 0.7	0.03 – 0.05	coccoid
15K	Římov res.	LimC4	0.4 – 0.7	0.02 – 0.04	ovoid
Rim6	Římov res.	LimA	0.9 – 1.3	0.12 – 0.25	solenoid
Rim8	Římov res.	LimA	0.5 – 0.7	0.03 – 0.05	solenoid
Rim11	Římov res.	LimB	0.5 – 0.8	0.03 – 0.05	short rods
Rim28	Římov res.	LimC5	0.4 – 0.6	0.03 – 0.04	coccoid
Rim42	Římov res.	LimC1	0.6 – 0.9	0.04 – 0.08	rods
Rim47	Římov res.	LimC4	0.5 – 0.7	0.04 – 0.06	coccoid
VIII-A6	Římov res.	LimC2	0.4 – 0.6	0.03 – 0.05	short rods
SP2	Seepromenade	LimC6	2.1 – 3.0	0.30 – 0.52	large solenoid
SP3	Seepromenade	LimA	0.7 – 1.1	0.06 – 0.10	solenoid
WS1	Wiestalstausee	LimC6	2.1 – 3.0	0.43 – 0.68	large solenoid

Table 2
The origin and morphological characteristics of isolated strains *Limnohabitans* spp. Note that shape classifications are only subjective.

Isolate	Lineage	Histidine	Proline	Leucine	Sorbitol	Acetate	Glucose	Glutamine	Glutamate
L. australis	LimA	-	-	-	w	+	w	-	-
L. curvus	LimA	-	-	-	w	+	+	w	-
Rim 11	LimB	+	+	+	-	nt	+	+	+
L. planktonicus	LimC1	+	+	+	+	+	+	+	+
2KL-16	LimC1	-	+	w	+	-	+	nt	nt
L. parvus	LimC2	-	+	-	w	-	+	w	+
2KL-17	LimC4	-	+	+	-	-	nt	+	+
B22-3vk	LimC4	w	+	+	+	nt	+	+	+
KL5	LimC5	-	-	-	-	-	nt	+	+
2KL-27	LimC5	-	-	-	w	+	w	nt	nt
2KL-3	LimC6	-	-	-	-	w	w	nt	nt
15K	LimC	-	-	-	-	+	-	nt	nt
2KL-1	LimC	-	+	w	-	+	+	nt	nt
2KL-5	LimC	-	-	-	-	-	+	-	-
G3-2	LimC	-	-	+	+	nt	+	w	+
KL6	LimC	w	+	+	-	nt	+	+	+
T6-5	LimC	+	+	+	+	nt	+	+	+
B10-3v	LimE	-	-	-	-	+	+	-	-

Table 3
Metabolic characteristics of newly isolated *Limnohabitans* spp. and described species. The characteristics of *L. australis*, *L. curvus*, *L. parvus* and *L. planktonicus* were taken from Hahn et al. (2010a, b) and Kasalický et al. (2010), respectively. +, positive; w, weak; -, negative growth; nt, not tested.

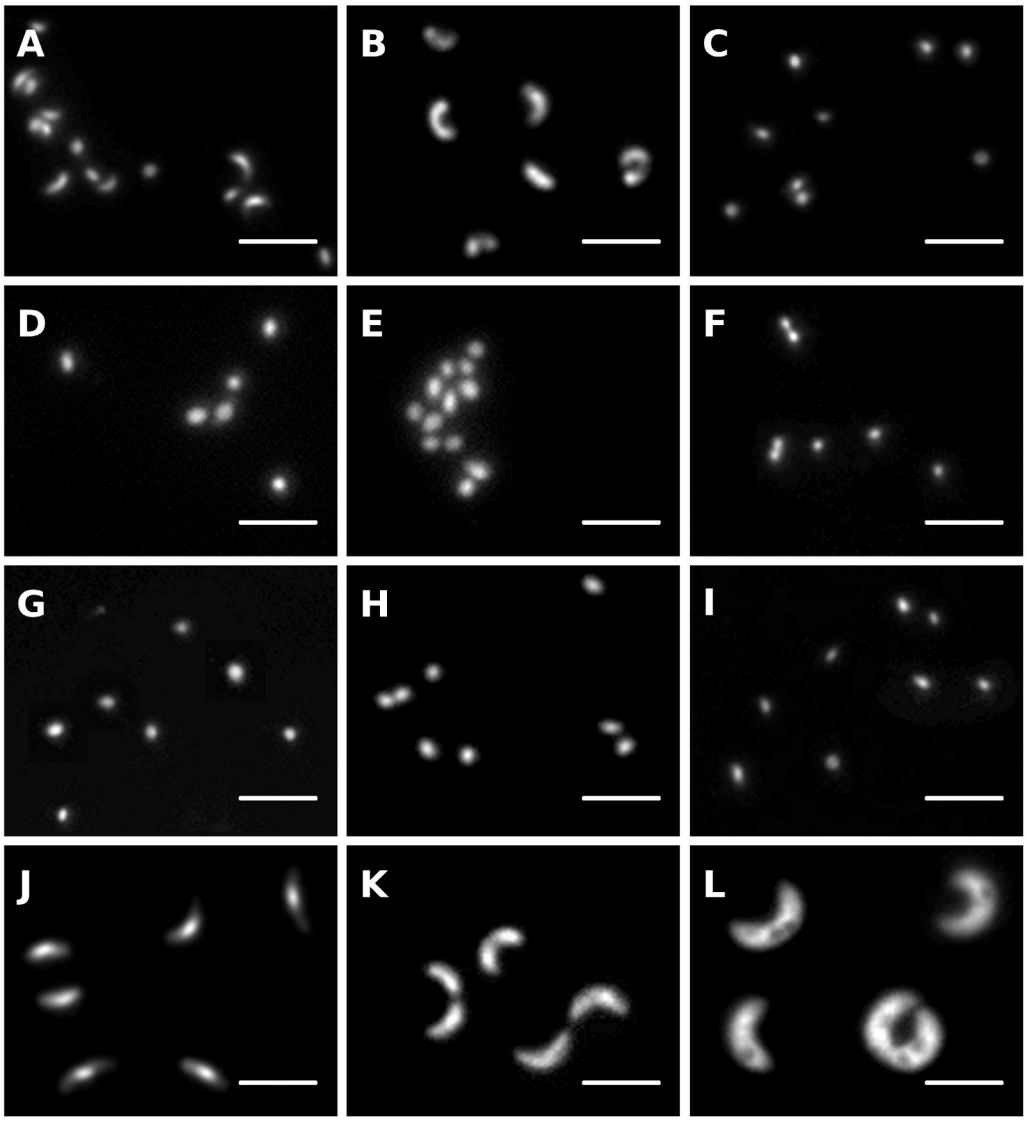
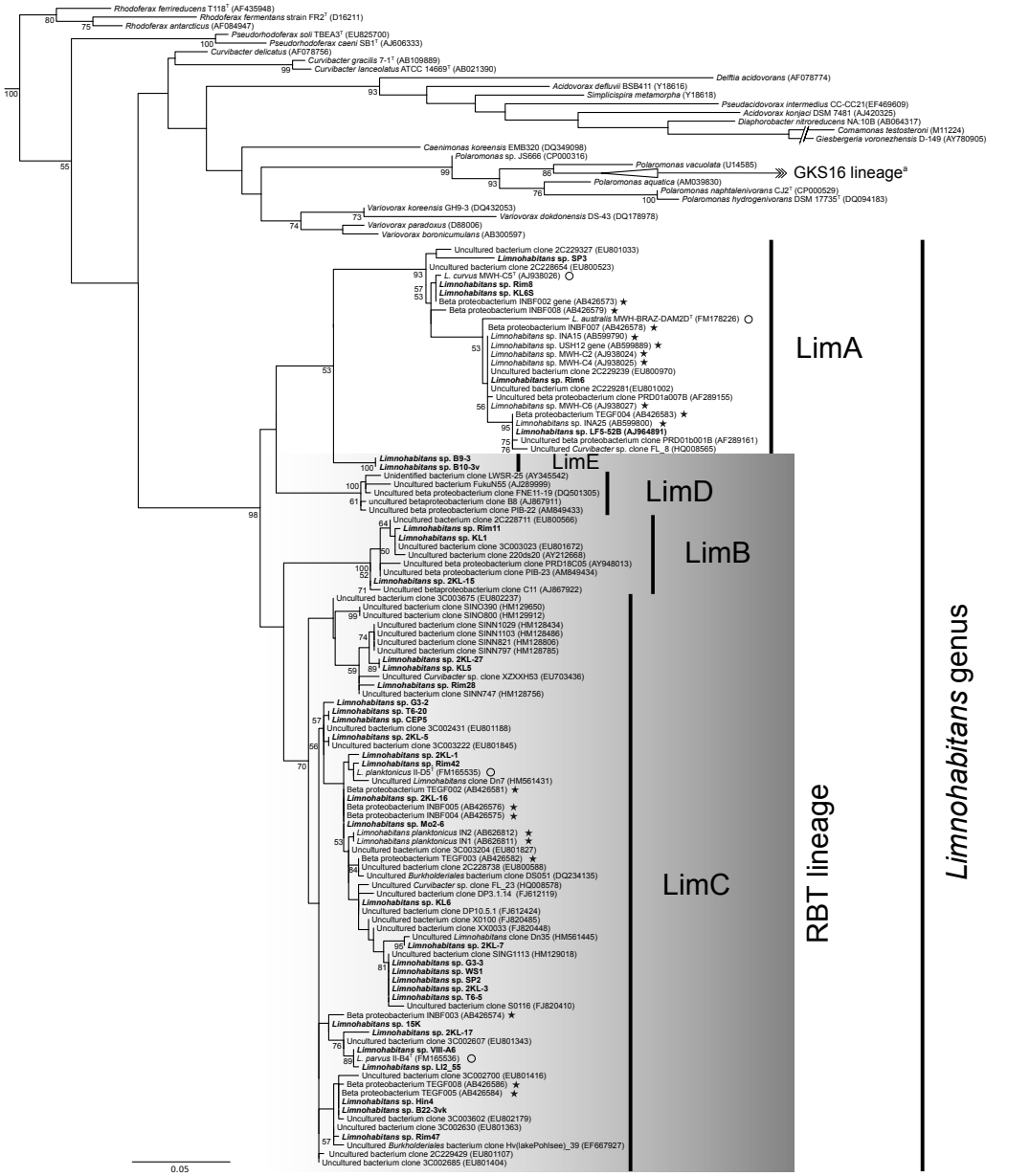


Figure 1
Basic morphotypes of isolated *Limnohabitans* spp. strains. (A, B) Lineage LimA - strains Rim8 and SP3, (B, C) lineage LimB - strains 2KL-15 and Rim11, (E - L) lineage LimC - strains 2KL-17 (LimC4), CEP5 (LimC3), 2KL-27 (LimC5), LI2-55 (LimC2, *L. parvus*), Hin4 (LimC4), 2KL-16 (LimC1, *L. planktonicus*), 2KL-1 (unaffiliated) and WS1 (LimC6). The strain-specific codes refer to the codes assigned to isolates in the overview Table 2. Microphotographs, 1000 x magnification, scale bar represents 2 μm.



a GKS16 lineage includes the type sequence GKS16 from the lake Gossenkohlesee (Austria) AJ224987 (Glöckner et al. 2001) and sequences AJ867749, AJ867919, AM849430, DQ228403, DQ521547, FJ849147, FJ849241, FJ849267, FJ849272, FJ849286, FJ849302, FJ849309, HQ327159, HQ327174, HQ327215, HQ327230, HQ595202, HQ622745

★ strains not described in this study

○ described species

Figure 2

Phylogenetic tree of isolated *Limnohabitans* spp. strains, environmental clones and described species based on 16S rRNA gene. GKS16 cluster is composed of the homonymous clone and other 19 environmental sequences. The consensus tree was constructed by Bayesian algorithm with 8 million generations, when 2 000 trees were removed as burnin. The scale bar correspond to 50 base substitutions per 100 nucleotide positions. Bootstrap values for Bayesian probability at the branching points are given. The tree was rooted by *Polynucleobacter necessarius* subsp. *asymbioticus*, *Ralstonia eutropha* and *Herbaspirillum putei*. Detailed description of used dataset is available in Suppl. Mat. Table 3.

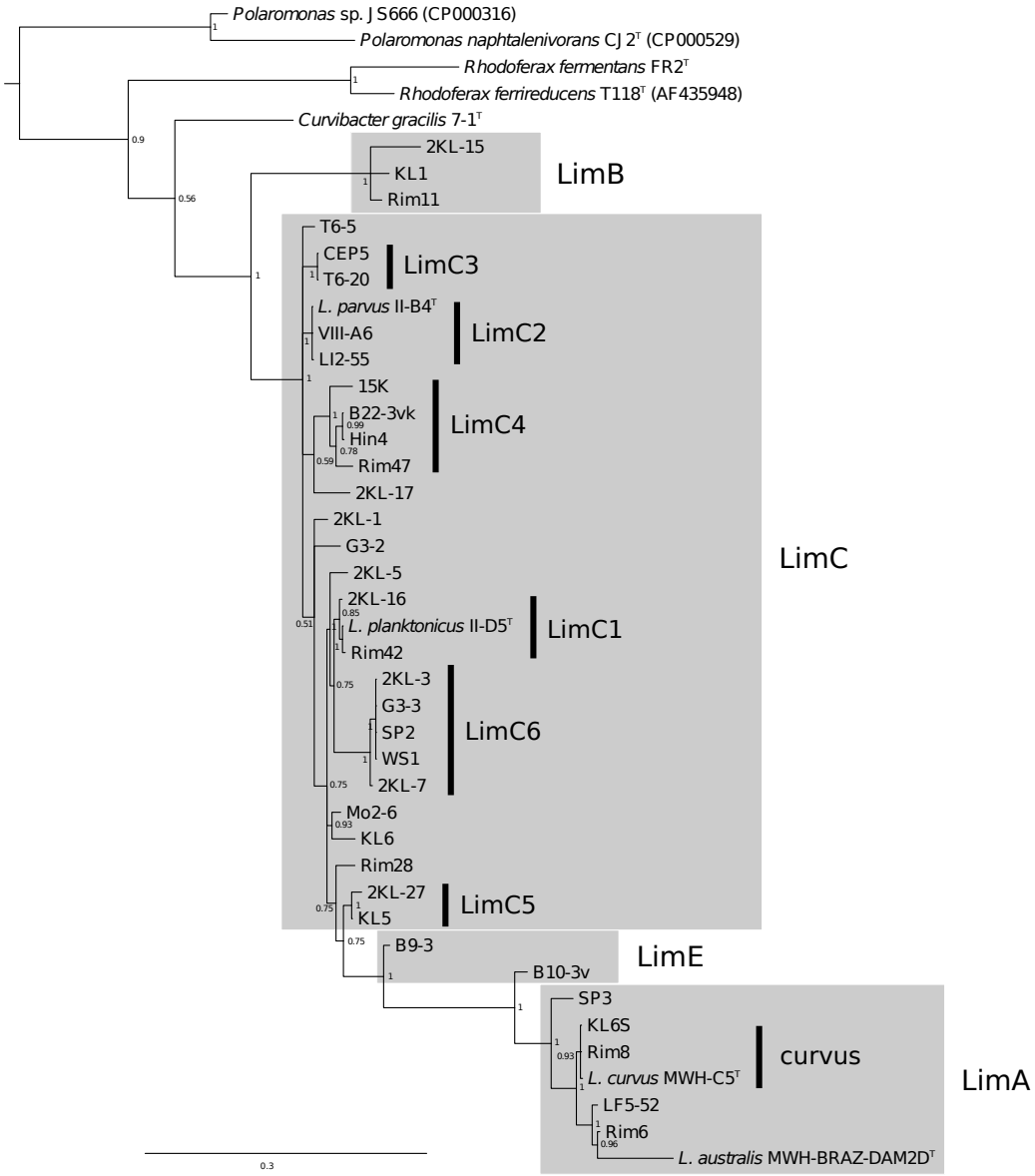


Figure 3
Phylogenetic tree of isolated *Limnohabitans* strains and closely related taxa based on both 16S rRNA gene and IGS1 sequence. The tree was constructed by Bayesian algorithm with 5 million generations, when 1.000 trees were removed as burnin. The scale bar correspond to 30 base substitutions per 100 nucleotide positions. Bootstrap values for Bayesian probability at the branching points are given. The tree was rooted by *Polynucleobacter necessarius* subsp. *asymbioticus*.

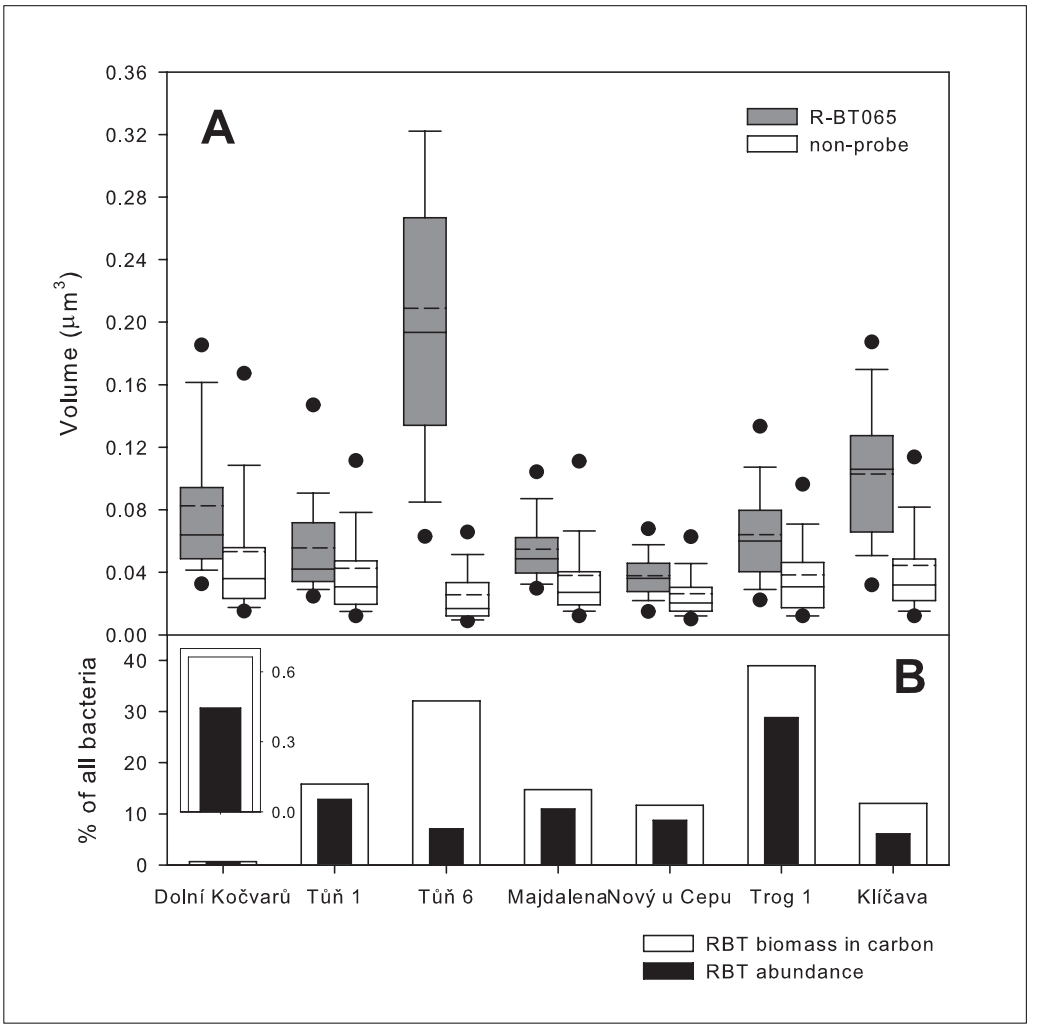


Figure 4
Volume of the R-BT065-positive cells compared to other bacteria (A) and their relative contribution in natural bacterial community (B). (A) Boxes represent 25% and 75% quartils, whiskers 5% and 95% quintiles, full circles outliers. Grey-the volume of the R-BT065 targeted cells, , white the rest of bacteria not targeted with the probe. Dashed lines represent means, whereas full lines are medians. (B) Relative proportions (%) of the RBT065 bacteria in total bacteria (black bar) and in total carbons biomass (white bar). Note that due to very low proportion of the RBT bacteria in humic pond Dolní Kočvarů there is also incorporated a fine-scale resolution insert.

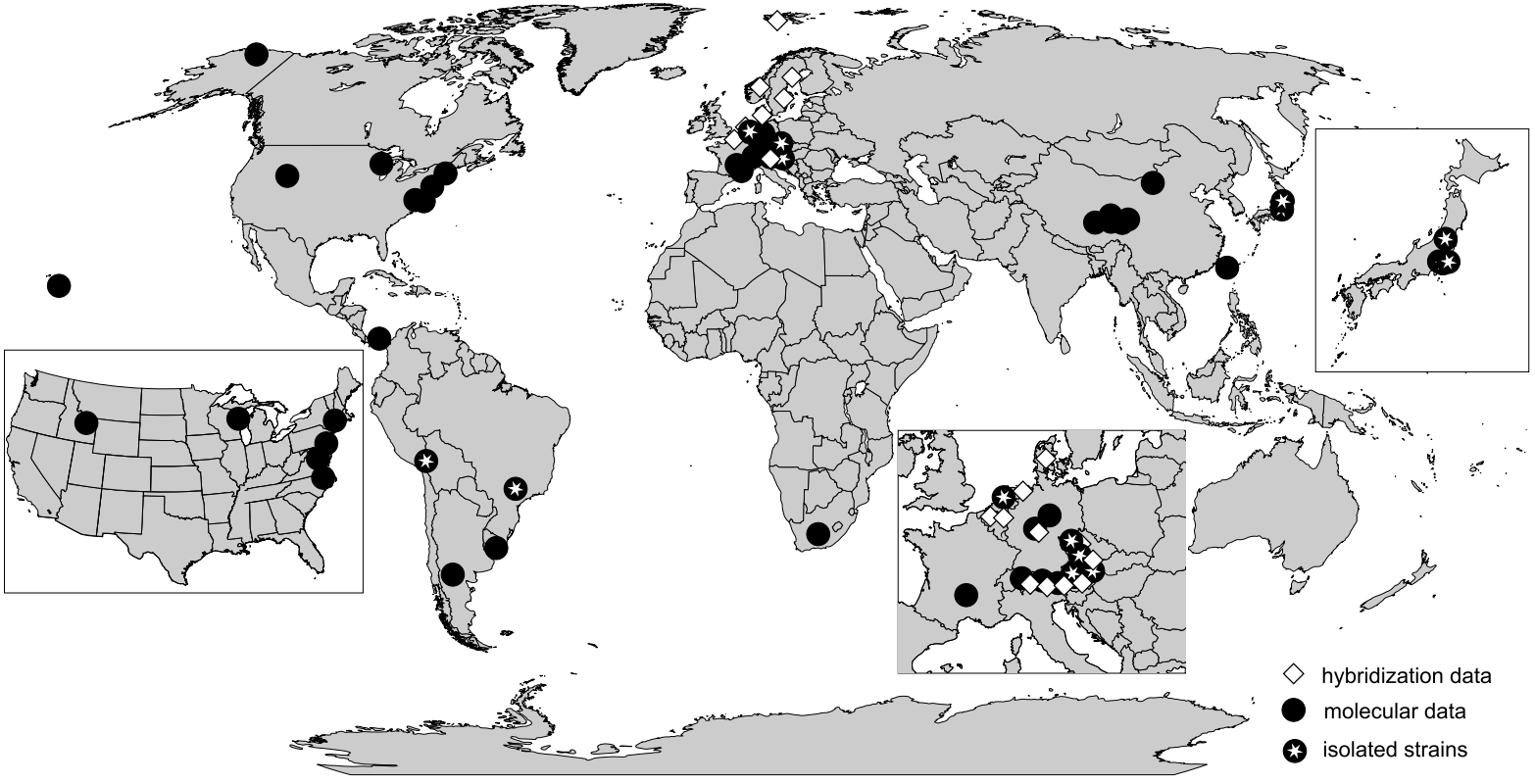


Figure 5
Biogeographic distribution of the *Limnohabitans* genus. Three data sources are distinguished: in situ hybridization (FISH, CARD-FISH, RLBH) (white diamonds); clone sequences (black dots); isolates (white stars).

Nucleotide differences \ Similarity					
	LimA	LimB	LimC	LimD	LimE
LimA	< 24, > 98 %	95.9 – 97.2 %	95.6 – 98.1 %	96 – 97.1 %	97.1 – 98.3 %
LimB	35 – 54	< 10, > 99 %	97 – 98.2 %	96.8 – 97.7 %	97 – 97.4 %
LimC	31 – 60	23 – 41	< 40, > 97 %	96.4 – 98 %	97.1 – 98.3 %
LimD	33 – 50	30 – 42	23 – 46	< 9, > 99 %	97 – 97.5 %
LimE	23 – 45	36 – 40	23 – 42	34 – 38	< 1, > 99 %

The grey backgrounded cells show max nucleotide diversity and min similarity within each lineage.

	Limnohabitans	*Rhodoferax*	*Curvibacter*	*Polaromonas*	GKS16 lineage	*Polynucleobacter*
Limnohabitans	< 60, > 95 %	92 – 97 %	94 – 97 %	93 – 96 %	93 – 96 %	86 – 87 %
Rhodoferax	51 – 67	< 38, > 96 %	93 – 97 %	91 – 96 %	90 – 95 %	86 – 89 %
Curvibacter	39 – 63	42 – 60	< 29, > 97 %	93 – 97 %	93 – 95 %	87 – 89 %
Polaromonas	65 – 94	60 – 95	45 – 80	< 48, > 96 %	96 – 98 %	86 – 89 %
GKS16 lineage	65 – 91	70 – 92	61 – 80	26 – 47	< 23, > 98 %	86 – 88 %
Polynucleobacter	172 – 192	154 – 173	164 – 166	161 – 176	168 – 173	< 12, > 98 %

The grey backgrounded cells marks the max nucleotide diversity and min similarity within each genus/cluster.
Data for *Rhodoferax* genus from only 3 strains.
Data for *Curvibacter* genus from only 3 strains.
Data for *Polaromonas* genus from only 5 strains.
Data for GKS16 lineage from only 20 strains.
Data for *Polynucleobacter* genus from only 2 strains.

Supplemental Table 1
Pairwise comparisons of aligned almost complete 16S rRNA gene sequences of newly isolated *Limnohabitans* strains and closely related environmental clones and other genera. The similarity is shown in the upper part, the lower part depicts the number of nucleotide differences between sequences.

Nucleotide differences \ Similarity				
	LimA	LimB	LimC	LimE
LimA (612–622 bp)	< 65, > 86 %	67 – 72 %	68 – 73 %	68 – 73 %
LimB (675–704 bp)	99 – 127	< 56, > 74 %	71 – 95 %	70 – 79 %
LimC (712–746 bp)	86 – 109	104 – 140	< 79, > 68 %	69 – 91 %
LimE (615–723 bp)	25 – 102	112 – 137	35 – 108	< 104, > 70 %

The grey backgrounded cells show max nucleotide diversity and min similarity within each lineage.
All *Limnohaitans* IGS1 sequences were retrieved during this work

Sequence similarity				
	LimA	LimB	LimC	LimE
*C. gracilis**	68 – 71 %	66 – 68 %	63 – 67 %	64 – 69 %
C. HmaUn_WGA71069	65 – 67 %	62 – 65 %	62 – 66 %	63 – 68 %
R. ferrireducens	64 – 67 %	62 – 64 %	60 – 63 %	61 – 67 %
*R. fermentans**	59 – 61 %	61 – 63 %	60 – 65 %	60 – 63 %
P. sp. JS666	61 – 63 %	58 – 61 %	54 – 59 %	57 – 63 %
P. naphtalenivorans	54 – 57 %	55 – 57 %	52 – 56 %	54 – 58 %
P.nec.asymbioticus	43 – 45 %	42 – 44 %	40 – 43 %	41 – 45 %

Nucleotide differences				
	LimA	LimB	LimC	LimE
*C. gracilis**	114 – 131	136 – 139	131 – 142	127 – 142
C. HmaUn_WGA71069	113 – 118	130 – 142	140 – 153	114 – 152
R. ferrireducens	127 – 132	142 – 149	151 – 166	127 – 165
*R. fermentans**	143 – 153	174 – 185	179 – 192	152 – 192
P. sp. JS666	139 – 144	142 – 156	146 – 160	141 – 154
P. naphtalenivorans	144 – 152	170 – 175	177 – 192	145 – 191
P.nec.asymbioticus	171 – 174	183 – 190	193 – 202	174 – 201

* IGS1 sequences of *R.fermentans* and *C.gracilis* retrieved during this work.

Nucleotide differences \ Similarity					
	Limnohabitans	*Curvibacter**	*Rhodoferax**	*Polaromonas*	*Polynucleobacter*
Limnohabitans	< 140, > 67 %	62 – 71 %	59 – 67 %	52 – 63 %	40 – 45 %
*Curvibacter**	113 – 153	< 127, > 66 %	60 – 77 %	54 – 63 %	43 – 46 %
*Rhodoferax**	127 – 192	89 – 154	< 83, > 77 %	55 – 64 %	38 – 41 %
Polaromonas	139 – 192	139 – 151	159 – 183	< 79, > 75 %	40 – 43 %
Polynucleobacter	171 – 202	171 – 195	181 – 199	161 – 172	-

The grey backgrounded cells marks the max nucleotide difference and min similarity within each genus.

Data for *Curvibacter* genus from only 2 strains.
Data for *Rhodoferax* genus from only 2 strains.
Data for *Polaromonas* genus from only 2 strains.
Data for *Polynucleobacter* genus from only 1 strain (Hahn et al. 2009).

Supplemental Table 2

Pairwise comparisons of complete complete 16S -23S rRNA intergenic spacer (IGS1) gene sequences of newly isolated *Limnohabitans* strains and closely related species. The similarity is shown in the upper part, the lower part depicts the number of mismatches between sequences.

Supplemental Table 3

Accession numbers of sequences from bacterial strains and environmental clones used in this work.

Isolated strains from *Limnohabitans* genus

Lineage	Strain description	Habitat	Country	Accession number	Authors
LimA	KL6S	Klíčava reservoir	Czech Republic	HE600679	this work
LimA	Rim6	Římov reservoir	Czech Republic	HE600681	this work
LimA	Rim8	Římov reservoir	Czech Republic	HE600682	this work
LimA	SP3	Seepromenade	Austria	HE600688	this work
LimB	2KL-15	Klíčava reservoir	Czech Republic	HE600664	this work
LimB	KL1	Klíčava reservoir	Czech Republic	HE600676	this work
LimB	Rim11	Římov reservoir	Czech Republic	HE600683	this work
LimC	15K	Římov reservoir	Czech Republic	HE600668	this work
LimC	2KL-1	Klíčava reservoir	Czech Republic	HE600660	this work
LimC	2KL-16	Klíčava reservoir	Czech Republic	HE600665	this work
LimC	2KL-17	Klíčava reservoir	Czech Republic	HE600666	this work
LimC	2KL-27	Klíčava reservoir	Czech Republic	HE600667	this work
LimC	2KL-3	Klíčava reservoir	Czech Republic	HE600661	this work
LimC	2KL-5	Klíčava reservoir	Czech Republic	HE600662	this work
LimC	2KL-7	Klíčava reservoir	Czech Republic	HE600663	this work
LimC	B22-3vk	Bagr pond	Czech Republic	HE600671	this work
LimC	CEP5	Nový u Cepu pond	Czech Republic	HE600672	this work
LimC	G3-2	Gosau 3	Austria	HE600673	this work
LimC	G3-3	Gosau 3	Austria	HE600674	this work
LimC	Hin4	Hintersee	Austria	HE600675	this work
LimC	KL5	Klíčava reservoir	Czech Republic	HE600677	this work
LimC	KL6	Klíčava reservoir	Czech Republic	HE600678	this work
LimC	Li2-55	Lake Mondsee	Austria	AJ664692	Hahn et al. 2010a, this work
LimC	Mo2-6	Lake Mondsee	Austria	HE600680	this work
LimC	Rim28	Římov reservoir	Czech Republic	HE600684	this work
LimC	Rim42	Římov reservoir	Czech Republic	HE600685	this work
LimC	Rim47	Římov reservoir	Czech Republic	HE600686	this work
LimC	SP2	Seepromenade	Austria	HE600687	this work
LimC	T6-20	Lužnice pond T6	Czech Republic	HE600690	this work
LimC	T6-5	Lužnice pond T6	Czech Republic	HE600689	this work
LimC	VIII-A6	Římov reservoir	Czech Republic	HE600691	this work
LimC	WS1	Westahlstausee	Austria	HE600692	this work
LimE	B9-3	Bagr pond	Czech Republic	HE600669	this work
LimE	B10-3v	Bagr pond	Czech Republic	HE600670	this work
LimA	*L. australis* MWH-BRAZ-DAM2D[T]		Brazil	FM178226	Hahn et al. 2010B; this work
LimA	*L. curvus* MWH-C5[T]	Lake Mondsee	Austria	AJ938026	Hahn et al. 2010a; this work
LimC	*L. parvus* II-B4[T]	Římov reservoir	Czech Republic	FM165536	Kasalicky et al. 2010; this work
LimC	*L. planktonicus* II-D5[T]	Římov reservoir	Czech Republic	FM165535	Kasalicky et al. 2010; this work
LimA	TEGF004	Lake Teganuma	Japan	AB426583	Watanabe et al. unpublished
LimA	INBF002	Lake Inbanuma	Japan	AB426573	Watanabe et al. unpublished
LimA	INBF007	Lake Inbanuma	Japan	AB426578	Watanabe et al. unpublished
LimA	INBF008	Lake Inbanuma	Japan	AB426579	Watanabe et al. unpublished
LimA	INA15	Lake Inawashiro	Japan	AB599790	Watanabe et al. unpublished
LimA	INA25	Lake Inawashiro	Japan	AB599800	Watanabe et al. unpublished
LimA	USH12	Lake Ushikunuma	Japan	AB599889	Watanabe et al. unpublished
LimC	TEGF002	Lake Teganuma	Japan	AB426581	Watanabe et al. unpublished
LimC	TEGF003	Lake Teganuma	Japan	AB426582	Watanabe et al. unpublished
LimC	TEGF005	Lake Teganuma	Japan	AB426584	Watanabe et al. unpublished
LimC	TEGF008	Lake Teganuma	Japan	AB426586	Watanabe et al. unpublished
LimC	IN1	Lake Inbanuma	Japan	AB626812	Watanabe et al. unpublished
LimC	IN2	Lake Inbanuma	Japan	AB626811	Watanabe et al. unpublished
LimC	INBF003	Lake Inbanuma	Japan	AB426574	Watanabe et al. unpublished
LimC	INBF004	Lake Inbanuma	Japan	AB426575	Watanabe et al. unpublished
LimC	INBF005	Lake Inbanuma	Japan	AB426576	Watanabe et al. unpublished

Bacterial species from Comamonadacea family

Strain description	Accession number	Authors
Acidovorax defluvii BSB411	Y18616	Schulze et al. 1999
Acidovorax konjac /DSM 7481	AJ420325	Willems A et al. 1992
Caenimonas koreensis EMB320	DQ349098	Ryu et al. 2008
Comamonas testosteroni	M11224	Yang et al. 1985
Curvibacter delicatus	AF078756	Wen et al. 1999
Curvibacter gracilis 7-1[T]	AB109889	Ding & Yokota 2004; this work
Curvibacter lanceolatus ATCC 14669[T]	AB021390	Anzai et al. 2000
Delftia acidovorans	AF078774	Wen et al. 1999
Diaphorobacter nitroreducens NA10B	AB064317	Tabrez Khan and Hiraishi 2001
Giesbergeria voronezhensis D-149	AY780905	Gerbovich et al. 2006
Polaromonas aquatica CCUG 39402[T]	AM039830	Kaempfer et al. 2006
Polaromonas hydrogenivorans DSM 17735[T]	DQ094183	Panikov and Sizova 2007
Polaromonas naphtalenivorans CJ2[T]	CP000529	Copeland et al. unpublished
Polaromonas sp. strain JS666	CP000316	Copeland et al. unpublished
Polaromonas vacuolata	U14585	Gosink and Staley 1995
Polynucleobacter necessarius subsp. *asymbioticus* QLW-P1DMWA-1[T]	CP000655	Copeland et al. unpublished
Pseudacidovorax intermedius CC-21	EF469609	Kaempfer et al. 2008
Pseudorhodoferax caeni SB1[T]	AJ606333	Bathe 2004
Pseudorhodoferax soli TBEA3[T]	EU825700	Bruland et al. 2009
Rhodoferax antarcticus ANT.BR	AF084947	Madigan et al. 2000
Rhodoferax fermentans FR2[T]	D16211	Hiraishi 1994, this work
Rhodoferax ferrireducens T118[T]	AF435948, CP000267	Finneran et al. 2003
Simplicispira metamorpha	NR_044941	Schulze et al. 1999
Variovorax boronicumulans	AB300597	Miwa et al. 2008
Variovorax dokdonensis DS-43	DQ178978	Yoon et al. 2006
Variovorax soli GH9-3	DQ432053	Kim et al. 2008
Variovorax paradoxus	D88006	Anzai et al. 2000

Environmental clones affiliated within the *Limnohabitans* genus

Lineage	Clone description	Habitat	Country	16S rRNA gene	Authors
LimA	PRD01a007B	Parker River	USA (Mass)	AF289155	Zwart et al. 2002
LimA	PRD01b001B	Parker River	USA (Mass)	AF289161	Zwart et al. 2002
LimA	2C228654	Delaware Bay, site GS11	USA (NJ)	EU800523	Shaw et al. 2008
LimA	H0051	Wuliangsuhai Lake - a shallow, hypertrophic freshwater lake	China (Inner Mongolia)	FJ820368	Feng et al. Unpublished
LimA	2C229239	Delaware Bay, site GS11	USA (NJ)	EU800970	Shaw et al. 2008
LimA	2C229281	Delaware Bay, site GS11	USA (NJ)	EU801002	Shaw et al. 2008
LimA	2C229327	Delaware Bay, site GS11	USA (NJ)	EU801033	Shaw et al. 2008
LimA	FL_8	Rio III reservoir, Cordoba	Argentina	HQ088565	Polverino et al. unpublished
LimB	C11-clone	Alpine Lake Joeri XIII	Switzerland	AJ867922	Yuhana unpublished
LimB	PIB-23	Pburger See	Austria	AM849434	Salcher et al. 2008
LimB	220ds20	water 20 m downstream of manure	USA (Ohio)	AY212668	Simpson et al. 2004
LimB	PRD18C05	Parker River	USA (Mass)	AY944013	Crump & Hobbie 2005
LimB	DP10.5.14	lake water	China	FJ612435	Li et al. unpublished
LimB	MI1G10	integrated lake epilimnion, Mirror Lake	USA (Wis)	FJ916099	Jones et al. 2009
LimB	ST11-17	Lake Stechlin	Germany	DQ501328	Allgaier & Grossart unpublished
LimB	YL139	Yellowstone Lake	USA (WY)	HM855504	Clingenpeel et al. Unpublished
LimB	ZS-4-312	lake epilimnion, 12.5 m depth, Lake Zurich	Switzerland	FM668013	Salcher et al. Unpublished
LimB	IRD18H02	Ipswich River	USA (MS)	AY947976	Crump & Hobbie 2005
LimB	2C228711	Delaware Bay, site GS11	USA (NJ)	EU800566	Shaw et al. 2008
LimB	3C003023	Chesapeake Bay, site GS12	USA (MD)	EU801672	Shaw et al. 2008
LimC	DS051	mangrove	Taiwan	DQ234135	Liao et al. 2007
LimC	Hv(lakePohlsee)_39	epithelium of *Hydra vulgaris* from Pohlsee	Germany	EF667927	Fraune & Bosch 2007
LimC	XZXX053	Eastern Tibetan Plateau, Lake Xinxinhai	China	EU703436	Xing et al. 2009
LimC	2C228738	Delaware Bay, site GS11	USA (NJ)	EU800588	Shaw et al. 2008
LimC	2C229429	Delaware Bay, site GS11	USA (NJ)	EU801107	Shaw et al. 2008
LimC	3C002431	Chesapeake Bay, site GS12	USA (MD)	EU801188	Shaw et al. 2008
LimC	3C002630	Chesapeake Bay, site GS12	USA (MD)	EU801363	Shaw et al. 2008
LimC	3C002685	Chesapeake Bay, site GS12	USA (MD)	EU801404	Shaw et al. 2008
LimC	3C002700	Chesapeake Bay, site GS12	USA (MD)	EU801416	Shaw et al. 2008
LimC	3C003204	Chesapeake Bay, site GS12	USA (MD)	EU801827	Shaw et al. 2008
LimC	3C003222	Chesapeake Bay, site GS12	USA (MD)	EU801845	Shaw et al. 2008
LimC	3C003602	Chesapeake Bay, site GS12	USA (MD)	EU802179	Shaw et al. 2008
LimC	3C003675	Chesapeake Bay, site GS12	USA (MD)	EU802237	Shaw et al. 2008
LimC	DP3.1.14	lake water	China	FJ612119	Li et al. unpublished
LimC	DP10.5.1	lake water	China	FJ612424	Li et al. unpublished
LimC	SINN1029	Tanggulha Lake	China (Tibet)	HM128434	Zhang & Liu unpublished
LimC	SINN1103	Tanggulha Lake	China (Tibet)	HM128486	Zhang & Liu unpublished
LimC	SINN747	Tanggulha Lake	China (Tibet)	HM128756	Zhang & Liu unpublished
LimC	SINN797	Tanggulha Lake	China (Tibet)	HM128785	Zhang & Liu unpublished
LimC	SINN821	Tanggulha Lake	China (Tibet)	HM128806	Zhang & Liu unpublished
LimC	SING1113	Kelike Lake	China (Tibet)	HM129018	Zhang & Liu unpublished
LimC	SINO390	Nam Co Lake	China (Tibet)	HM129650	Zhang & Liu unpublished
LimC	SINO800	Nam Co Lake	China (Tibet)	HM129912	Zhang & Liu unpublished
LimC	Dn7	digestive tract of *Daphnia magna*	Germany	HM561431	Freese & Schink 2011
LimC	FL_23	Rio III reservoir, Cordoba	Argentina	HQ088578	Polverino et al. unpublished
LimC	S0116	Wuliangsuhai Lake, shallow hypertrophic lake	China (Inner Mongolia)	FJ820410	Feng et al. unpublished
LimC	XX0033	Wuliangsuhai Lake, shallow hypertrophic lake	China (Inner Mongolia)	FJ820448	Feng et al. unpublished
LimC	X0100	Wuliangsuhai Lake, shallow hypertrophic lake	China (Inner Mongolia)	FJ820485	Feng et al. unpublished
LimC	Dn35	digestive tract of *Daphnia magna*	Germany?	HM561445	Freese & Schink 2011
LimC	3C002607	Chesapeake Bay, site GS12	USA (MD)	EU801343	Shaw et al. 2008
LimD	FukuN55	Lake Fuchskuhle, north east basin	Germany	AJ289999	Glöckner et al. 2000
LimD	B8-clone	Alpine Lake Joeri XIII	Switzerland	AJ867911	Yuhana unpublished
LimD	PIB-22	Pburger See	Austria	AM849433	Salcher et al. 2008
LimD	A07-39-BAC	water column Marathonas Reservoir	Greece	GQ340078	Kormas et al. Unpublished
LimD	ADK-CSe02-53	acid-impacted Adirondack lake, Cascade lake (pH = 6.95)	USA (New York)	EF520468	Percent et al. 2008
LimD	TLM05/TLMdgge10	Toolik Lake main station at 3 m depth	USA (Alaska)	AF534429	Crump et al. 2003
LimD	LWSR-25	Lake Wai'ele'ele	USA (Hawaii)	AY345542	Donachie et al. unpublished
LimD	PIB-25	Pburger See	Austria	AM849436	Salcher et al. 2008
LimD	YL098	Yellowstone Lake	USA (WY)	HM856464	Clingenpeel et al. Unpublished
LimD	2C229073	Delaware Bay, site GS11	USA (NJ)	EU800848	Shaw et al. 2008
LimD	2C229453	Delaware Bay, site GS11	USA (NJ)	EU801123	Shaw et al. 2008
LimD	5C231162	Lake Gatun	Panama	EU803573	Shaw et al. 2008
LimD	2C229321	Delaware Bay, site GS11	USA (NJ)	EU801028	Shaw et al. 2008
LimD	RNE11-19	Lake Grosse Fuchskuhle, NE compartment	Germany	DQ501305	Allgaier & Grossart unpublished

Environmental clones affiliated within the GKS16 lineage

Lineage	Clone description	Habitat	Country	16S rRNA gene	Authors
GKS16	GKS16	Lake Gossenkoellesee	Austria	AJ224987	Glöckner et al. 2000
GKS16	JFJ-ICE-Bact-06	melted-ice water, Moenchsjoch, Alpine Jungfraujoch	Switzerland	AJ867749	Yuhana unpublished
GKS16	clone C7	Alpine Lake Joeri XIII	Switzerland	AJ867919	Yuhana unpublished
GKS16	PIB-19	Pburger See	Austria	AM849430	Salcher et al. 2008
GKS16	JEG_e1	John Evans Glacier	Canada	DQ228403	Skidmore et al. 2005
GKS16	ANTLV7_H07	Lake Vida ice cover, McMurdo Dry Valleys, Southern Victoria Land	Antarctica	DQ521547	Mosier et al. 2007
GKS16	EpiNCB23	arctic stream epilithon, Noatak National Preserve	USA (Alaska)	FJ849147	Larouche et al. unpublished
GKS16	EpiUMA57	arctic stream epilithon, Noatak National Preserve	USA (Alaska)	FJ849241	Larouche et al. unpublished
GKS16	EpiUMB15	arctic stream epilithon, Noatak National Preserve	USA (Alaska)	FJ849267	Larouche et al. unpublished
GKS16	EpiUMB2	arctic stream epilithon, Noatak National Preserve	USA (Alaska)	FJ849272	Larouche et al. unpublished
GKS16	EpiUMB34	arctic stream epilithon, Noatak National Preserve	USA (Alaska)	FJ849286	Larouche et al. unpublished
GKS16	EpiUMB49	arctic stream epilithon, Noatak National Preserve	USA (Alaska)	FJ849302	Larouche et al. unpublished
GKS16	EpiUMB55	arctic stream epilithon, Noatak National Preserve	USA (Alaska)	FJ849309	Larouche et al. unpublished
GKS16	TP-Snow-5	snow	China (Tibet)	HQ327159	Zhao & Ma unpublished
GKS16	TP-Snow-20	snow	China (Tibet)	HQ327174	Zhao & Ma unpublished
GKS16	TP-Snow-74	snow	China (Tibet)	HQ327214	Zhao & Ma unpublished
GKS16	TP-Snow-75	snow	China (Tibet)	HQ327215	Zhao & Ma unpublished
GKS16	TP-Snow-93	snow	China (Tibet)	HQ327230	Zhao & Ma unpublished
GKS16	IC3028	ice core from Austre Lovenbreen glacier, Ny-Alesund	Norway (Svalbard)	HQ595202	Zheng & Zhang unpublished
GKS16	IC4073	ice core from Austre Lovenbreen glacier, Ny-Alesund	Norway (Svalbard)	HQ622745	Zheng & Zhang unpublished

CURRICULUM VITAE

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2000 – 2003: Bc. degree in Biology, Faculty of Biological Sciences, University of South Bohemia, České Budějovice.

Professional experience:

2006 – present: Ph.D. student, Institute of Hydrobiology, Biology Centre of ASCR v.v.i.; České Budějovice, Czech Republic.

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Abroad stays and fellowships:

2004 – 2010: Laboratoire Océanographique de Villefranche sur mer, France; Dr. Markus Weinbauer and Dr. Antoine Sciandra (three months)

2007: Field expedition on the Volga River, Beloe Lake, Šeksninsk and Rybinsk Reservoirs, Institute of Biology of Inland Waters, Russian Academy of Sciences, Borok, Russia; Dr. Kopylov (two weeks)

2007 – 2012: Institut für Limnologie OAW, Austria; Dr. Martin Hahn (several short-term stays)

2011: Universität Konstanz, Germany; Dr. Heike Freese (two weeks)

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2007 – 2009: Supervisor of Bachelor degree student Kateřina Pěchotová [Factors influencing the abundance of bacterial group *Limnohabitans* and its ecology in eutrophic environment. Bachelor thesis], Department of Biology of Ecosystems, Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic.

2007 – 2009: Teaching assistant, Faculty of Science, University of South Bohemia, České Budějovice. *Microbiology*. (practical seminars).

2008: Teaching assistant, Faculty of Science, University of South Bohemia, České Budějovice. *Special limnological methods* and *Modern limnological methods* (practical seminars).

2008 – 2010: Teaching assistant, Faculty of Science, University of South Bohemia, České Budějovice. *Seminar for master degree students* (seminars).

2006 – 2011: Teaching assistant, Faculty of Science, University of South Bohemia, České Budějovice. *Field practice "Vomáčka"– hydrobiology, demonstration of algae and zooplankton.*

Grant and project participation:

GAČR 206/08/0015 (2008 – 2012) (Karel Šimek) *"Ecophysiological traits and grazing- and virus-induced mortality of bacterial strains representing major bacterioplankton groups in a reservoir"*. Member of the research team.

GAČR P504/10/0566 (2010 – 2012) (Jan Jezbera) *"Distribution, phylogeography and within-taxon ecological differentiation of Limnohabitans cluster and Polynucleobacter necessarius subsp. asymbioticus."* Member of the research team.

GAAV P504/10/1534 (2008 – 2011) (Karel Horňák) *"Influence of phytoplankton on bacterial community composition and activity under varying trophic status"*. Member of the research team.

International joint research project: **KONTAKT** (2007-08) (Karel Šimek) *"Eco-physiological characteristics of two important groups of Betaproteobacteria abundant in freshwater bacterioplankton"*. Co-Applicant.

International Scientific and Technical Cooperation: **KONTAKT** (2009-10) (Jan Jezbera) *"Biogeography and within-taxon ecological differentiation of Limnicola planktonicus and Polynucleobacter necessarius (Betaproteobacteria)"*. Co-Applicant.

Publications:

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