



Aerobic Anoxygenic Photosynthesis Is Commonly Present within the Genus *Limnohabitans*

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ABSTRACT The genus *Limnohabitans* (*Comamonadaceae*, *Betaproteobacteria*) is a common and a highly active component of freshwater bacterioplanktonic communities. To date, the genus has been considered to contain only heterotrophic species. In this study, we detected the photosynthesis genes *pufLM* and *bchY* in 28 of 46 strains from three *Limnohabitans* lineages. The *pufM* sequences obtained are very closely related to environmental *pufM* sequences detected in various freshwater habitats, indicating the ubiquity and potential importance of photoheterotrophic *Limnohabitans* in nature. Additionally, we sequenced and analyzed the genomes of 5 potentially photoheterotrophic *Limnohabitans* strains, to gain further insights into their phototrophic capacity. The structure of the photosynthesis gene cluster turned out to be highly conserved within the genus *Limnohabitans* and also among all potentially photosynthetic *Betaproteobacteria* strains. The expression of photosynthetic complexes was detected in a culture of *Limnohabitans planktonicus* II-D5^T using spectroscopic and pigment analyses. This was further verified by a novel combination of infrared microscopy and fluorescent *in situ* hybridization.

IMPORTANCE The data presented document that the capacity to perform anoxygenic photosynthesis is common among the members of the genus *Limnohabitans*, indicating that they may have a novel role in freshwater habitats.

KEYWORDS FISH, IR microscopy, *Limnohabitans*, bacteriochlorophyll, *bchY*, freshwater *Betaproteobacteria*, photosynthetic bacteria, *pufM*

Aerobic anoxygenic phototrophs (AAPs) are bacteria that supplement their mostly heterotrophic metabolism with light energy harvested using bacteriochlorophyll-containing reaction centers (1). AAPs constitute 1 to 11% of the total prokaryotes in the euphotic zone of the world's oceans (2–4), while they may represent up to 34% in more eutrophic environments such as shelf seas and estuaries (5, 6). AAPs also represent a common component of freshwater microbial communities. Large numbers of AAPs were reported for temperate lakes (7–9), peat bogs (10), and rivers (11), frequently correlating with the trophic state and temperature (12). Cultivation-independent approaches showed that AAPs represent a phylogenetically heterogeneous group that includes different clades of *Alphaproteobacteria*, *Gammaproteobacteria*, and *Betaproteobacteria* (13, 14). While the first two clades dominate the marine environment, *Betaproteobacteria* strains were documented in brackish and freshwater environmental samples. In particular, a large portion of *pufM* sequences were affiliated with a purple nonsulfur bacterium, *Rhodospirillum rubrum* (*Comamonadaceae*), as the closest cultured relative (15–18). However, *Rhodospirillum* spp. do not usually constitute an important fraction of freshwater bacterioplankton, in contrast to members of the closely related genus *Limnohabitans* (19).

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The genus *Limnohabitans* represents an average of 12% of freshwater bacterioplankton, with global distribution in a broad range of habitats (20). Five lineages were differentiated within the genus *Limnohabitans* (21), but only four (called the “R-BT cluster”) can be targeted by fluorescence *in situ* hybridization (FISH) with the R-BT065 probe (22). The members of the R-BT cluster share a high growth potential, have relatively large mean cell volumes (0.05 to 0.16 μm^3), compared to typical species of bacterioplankton, and are subject to high levels of grazing by protists (21, 23, 24). These ecophysiological traits underline their importance in carbon flow through freshwater food webs (25). The members of four lineages of the genus *Limnohabitans* are available as cultivated strains; however, due to the lack of any pigmentation, this genus has been considered to be heterotrophic (26–28).

Since the genus *Limnohabitans* was originally delineated from the genus *Rhodofera* (it encompasses species formerly affiliated with the “*Rhodofera* sp. BAL47” cluster) (29), we speculated that the environmental betaproteobacterial *pufM* sequences may actually originate from the genus *Limnohabitans*. To verify our hypothesis, we investigated the phototrophic potential in our collection of 46 *Limnohabitans* strains isolated from the epilimnion of various freshwater lakes (21).

RESULTS

Detection of photosynthesis genes in *Limnohabitans* strains. To identify potentially phototrophic strains, we screened our collection of 46 *Limnohabitans* strains for the presence of *pufLM* genes (encoding large and small subunits of the bacterial photosynthesis reaction centers) and the *bchY* gene (encoding a subunit of bacteriochlorophyll reductase Y), using PCR. The *pufLM* genes were detected in 28 of 46 examined strains from A, B, and C *Limnohabitans* lineages (Fig. 1), but amplification of the bacteriochlorophyll reductase gene *bchY* was positive for only 22 *pufLM*-positive strains. Since phototrophic organisms always contain the *pufLM* and *bchY* genes, this indicates that the *bchY* primers failed to amplify certain forms of *bchY* genes (see references 30 and 31).

Phylogenetic analyses of the amino acid sequences of both the *pufM* (Fig. 2A) and *bchY* (Fig. 2B) genes showed a grouping of all *Limnohabitans* sequences close to betaproteobacterial sequences of *Hydrogenophaga*, *Rhodofera*, *Roseateles*, *Rubrivivax*, and *Methyloversatilis*. In contrast, both sets of *Limnohabitans* sequences were found to be distant from two sequences of *Polynucleobacter* genus members, i.e., *Polynucleobacter duraquae* strain MWH-MoK4 and *Polynucleobacter* sp. strain QLW-P1FAT50C-4, and marine *Alphaproteobacteria* members, i.e., genera *Dinoroseobacter*, *Rhodobacter*, and *Roseobacter*.

The studied *Limnohabitans* strains clustered in three phylogenetic groups (Fig. 2). All three groups were characterized by high within-group similarities of their sequences (>94% for the amino acid sequences and >83% for the nucleic acid sequences) for both *pufM* and *bchY* genes. Group I contained 24 strains from the LimC lineage (Fig. 1) (see reference 21) and 2 LimA strains (JirII-29 and JirII-31). Group II consisted of LimB members (strains Rim11, Hippo4, and 103DPR2), with <93% amino acid sequence similarities with group I. The most distant group, group III, included sequences from strains affiliated with LimE (strain B9-3) and LimC (strains 15K and 2KL-51) lineages. The amino acid sequence similarities of group III members with respect to both other groups were <81%. The phylogenetic distance of group III from the other two groups was supported by the fact that two *Hydrogenophaga* sequences separated *Limnohabitans* group III from groups I and II. The GC content of *pufM* sequences was highest for group I (57.6 to 63.3%) and lower for groups II and III (54.2 to 55% and 53.6 to 56.3%, respectively).

Phylogenetic analyses of *pufM* genes grouped 20 uncultured bacterial clones and 1 fosmid (all retrieved from public databases) within the genus *Limnohabitans* (Fig. 2A). The fosmid and 9 clones were placed directly within group I and 4 clones were closely related to group III, while none belonged directly to group II.

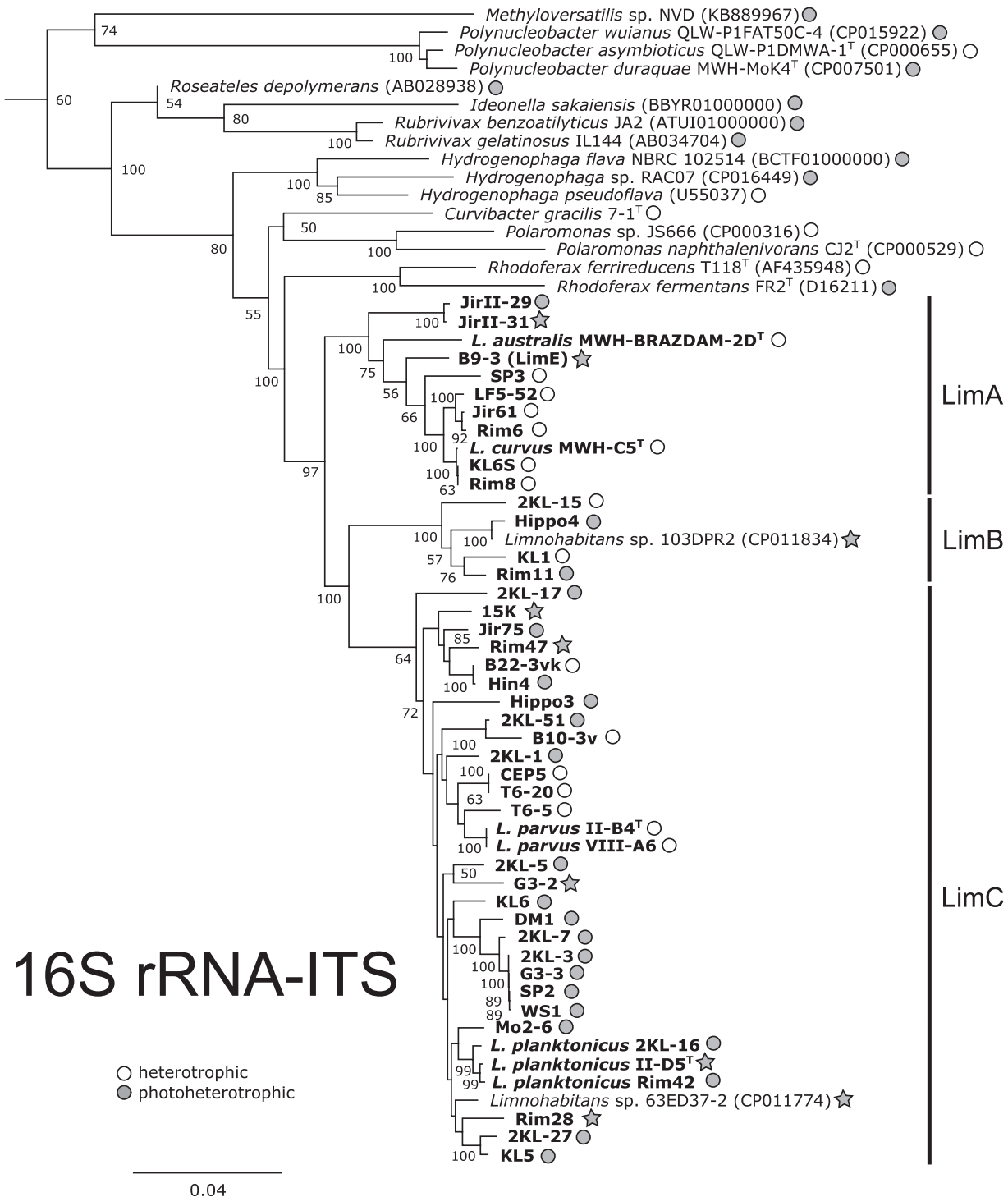


FIG 1 Phylogenetic tree with the metabolic diversification of the genus *Limnohabitans* and selected *Betaproteobacteria* species. The phylogenetic tree was calculated from sequences of the small ribosomal subunits and the internal transcribed spacer (ITS) region using Bayesian inference with 5 million generations. The metabolic type is indicated by color, as follows: gray, photoheterotrophy (*pufLM* presence); white, heterotrophy (none of the selected genes was detected). The stars indicate strains with available genomes.

Conserved one-cluster photosynthesis gene organization. We performed whole-genome sequencing of 5 selected strains, i.e., *Limnohabitans planktonicus* strain II-D5^T and *Limnohabitans* sp. strains 15K, B9-3, JirII-31, and G3-2, to obtain more information about the photosynthesis gene organization of the *pufM*-positive strains (Table 1). We also analyzed previously sequenced genomes of *Limnohabitans* sp. strains Rim28 and

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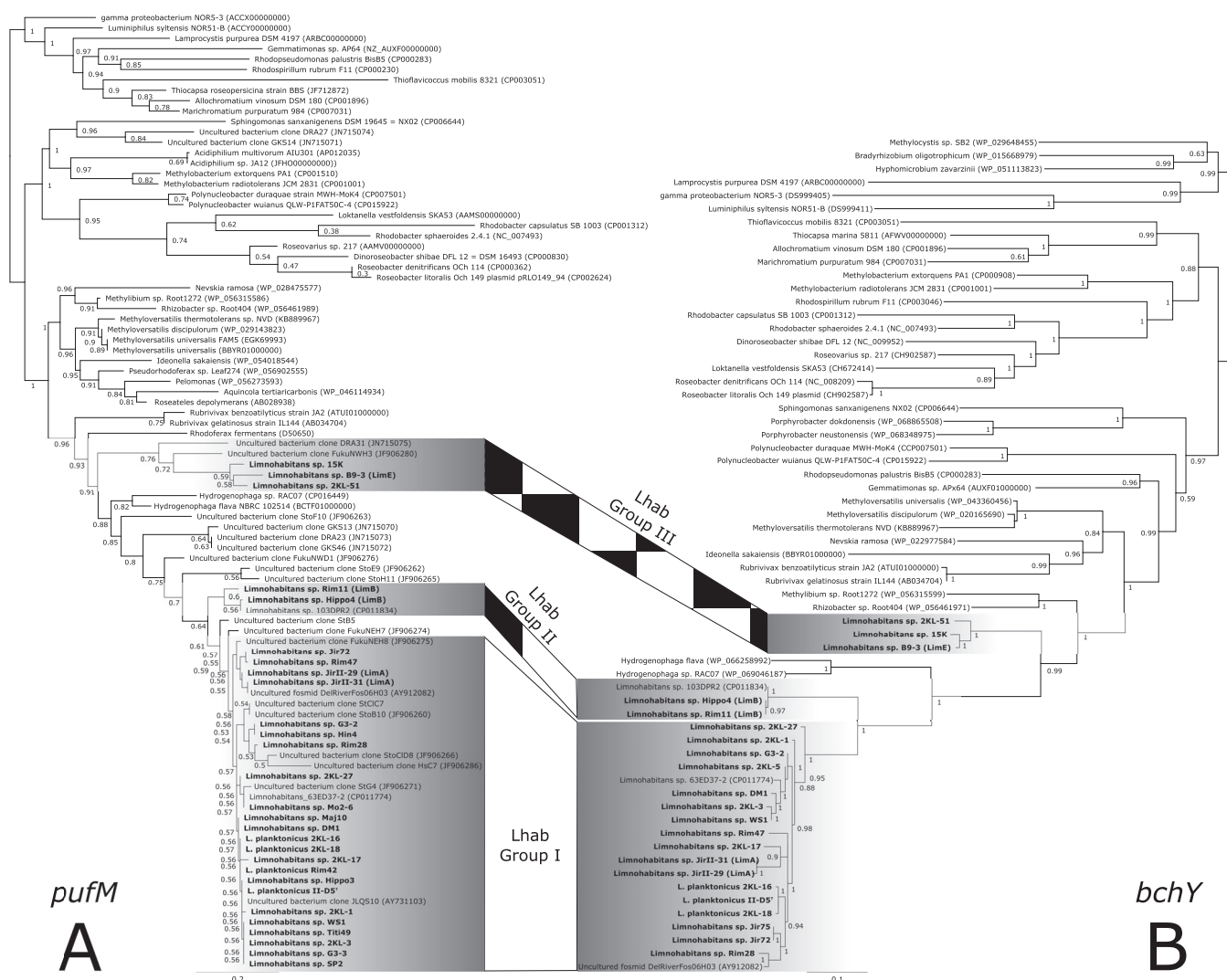


FIG 2 Phylogeny of phototrophy-related genes. The *pufM* gene (A) and *bchY* gene (B) phylogeny of the newly obtained *Limnohabitans* sequences (in bold) was determined with sequences retrieved from the NCBI RefSeq database. The *pufM* analysis allowed affiliation of environmental *pufM* clones from lakes in northern Germany (17), lakes in Austria (37), and the estuarine region of the East China Sea (67) with the genus *Limnohabitans*. The tree was calculated from amino acid alignments using the maximum likelihood approach with general time-reversible plus invariant plus γ distribution and χ^2 statistics for branch support.

Rim47. All of the sequenced species contained photosynthesis genes (from 42 to 47 genes) organized in a photosynthesis gene cluster (PGC). The main part of the PGC in *Limnohabitans* is formed by two conserved regions, with the first two regulatory genes being oriented in opposite directions (Fig. 3). The first conserved region contains the genes *crtEF*, *bchCXYZ*, *pufBALMC*, and *crtADC* and the second contains *ppsR*, *ppaA*, *bchFNBHLM*, *pucC*, *puhABC*, *acsF*, and *puhE* (Fig. 3). Both regions are also highly conserved in genomes of the *Betaproteobacteria* members *Methyloversatilis* sp. strain NVD, *Rubrivivax gelatinosus*, and *Rubrivivax benzoatilyticus* and even in the *Alphaproteobacteria* members *Roseobacter litoralis* and *Rhodobacter sphaeroides*. In contrast to the aforementioned genomes, the *Limnohabitans* PGC has a *crtIB-bluF* gene set located next to *puhE*. The whole PGC contained another three sets of genes, i.e., genes encoding bacteriochlorophyll synthase (*bchGP*) and bacteriochlorophyll reductase (*bchIDO*) and regulatory genes (*tspO*, *ppaA*, *cvrA/nhaP*, and *F420*), which are of variable order and composition (detailed in Table S1 in the supplemental material). Genomes of *Limnohabitans* sp. strains 15K and B9-3 have an opposite orientation of the second conserved region, compared to the genomes of strains II-D5, Jir11-31, Rim28, Rim47, and

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TABLE 1 Characteristics of *Limnohabitans* genomes and PGC clusters

Characteristic	Strain JirII-31	Strain 15K	Strain II-D5 ^T	Strain G3-2	Strain B9-3
<i>Limnohabitans</i> lineage	LimA	LimC	LimC	LimC	LimE
Genome information					
Genome size (Mb)	3.48	3.55	4.74	3.25	3.40
Mean sequencing coverage (fold)	200	210	60	250	220
No. of contigs	44	15	7	25	23
G+C content (%)	57.8	58.2	59.4	60.1	56.5
No. of protein-coding genes	3,368	3,342	4,273	3,025	3,174
Coding region (% of bp)	94.7	93.0	85.5	92.5	94.1
No. of rRNA operons	2	2	4	2	2
No. of tRNA genes	44	43	45	41	42
Photosynthesis gene cluster					
PGC length (kb)	48.0	54.2	48.7	45.6	51.1
PGC type	I	II	I	I	II
Outer antenna (PucAB)	+	-	+	+	-
L-POR	-	+	-	-	-

G3-2. In strain II-D5^T, the PGC is split into two parts (between *pucC* and *bluF*), separated by six genes related to arginine export.

All sequenced *Limnohabitans* strains contain only an aerobic form of Mg-protoporphyrin cyclase (*acsF* gene product) in their genomes, whereas the anaerobic form (*bchE* gene product) is absent. The presence of the *pufC* gene in all strains indicates that *Limnohabitans* strains use anoxygenic reaction centers with a tetraheme cytochrome *c* subunit. In contrast, the *pufX* gene, which is frequently present among phototrophic *Rhodobacterales* strains, has not been found in any *Limnohabitans* strains. The PGC also contained the *crtA* gene, which encodes the spheroidene monooxygenase (Fig. 3) that converts spheroidene into spheroidenone in the final step of spheroidenone biosynthesis. *Limnohabitans* sp. strains 15K and B9-3 were the only strains lacking *pucA* and *pucB* genes, which encode the subunits of the outer light-harvesting complex that enlarge the optical cross section of photosynthesis reaction centers.

Light-dependent protochlorophyllide oxidoreductase in *Limnohabitans*. A very interesting feature is the presence of light-dependent protochlorophyllide oxidoreductase (L-POR) in the genome of *Limnohabitans* sp. strain 15K. The percent identity between the *Limnohabitans* sp. strain 15K L-POR and the closest homologs was low, at 82.3%. The *Limnohabitans* L-POR sequence clustered together with sequences of the

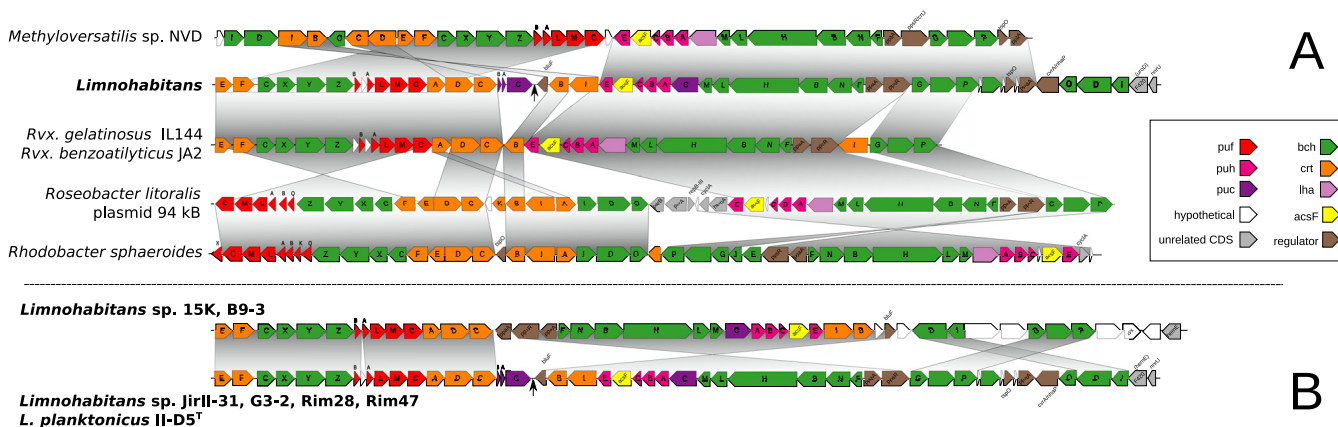


FIG 3 Photosynthesis gene cluster organization. (A) The PGCs in *Limnohabitans*, *Methyloversatilis*, and *Rubrivivax* (all *Betaproteobacteria*) and in *Rhodospirillum rubrum* and *Roseobacter* (both *Alphaproteobacteria*) were compared. (B) The organization of PGC in *Limnohabitans* sp. strain 15K and B9-3 differed from that in other *Limnohabitans* genomes. Photosynthesis genes were annotated according to reference 13, and the full annotation is available in Table S1 in the supplemental material. *Rvx.*, *Rubrivivax*; CDS, coding DNA sequence.

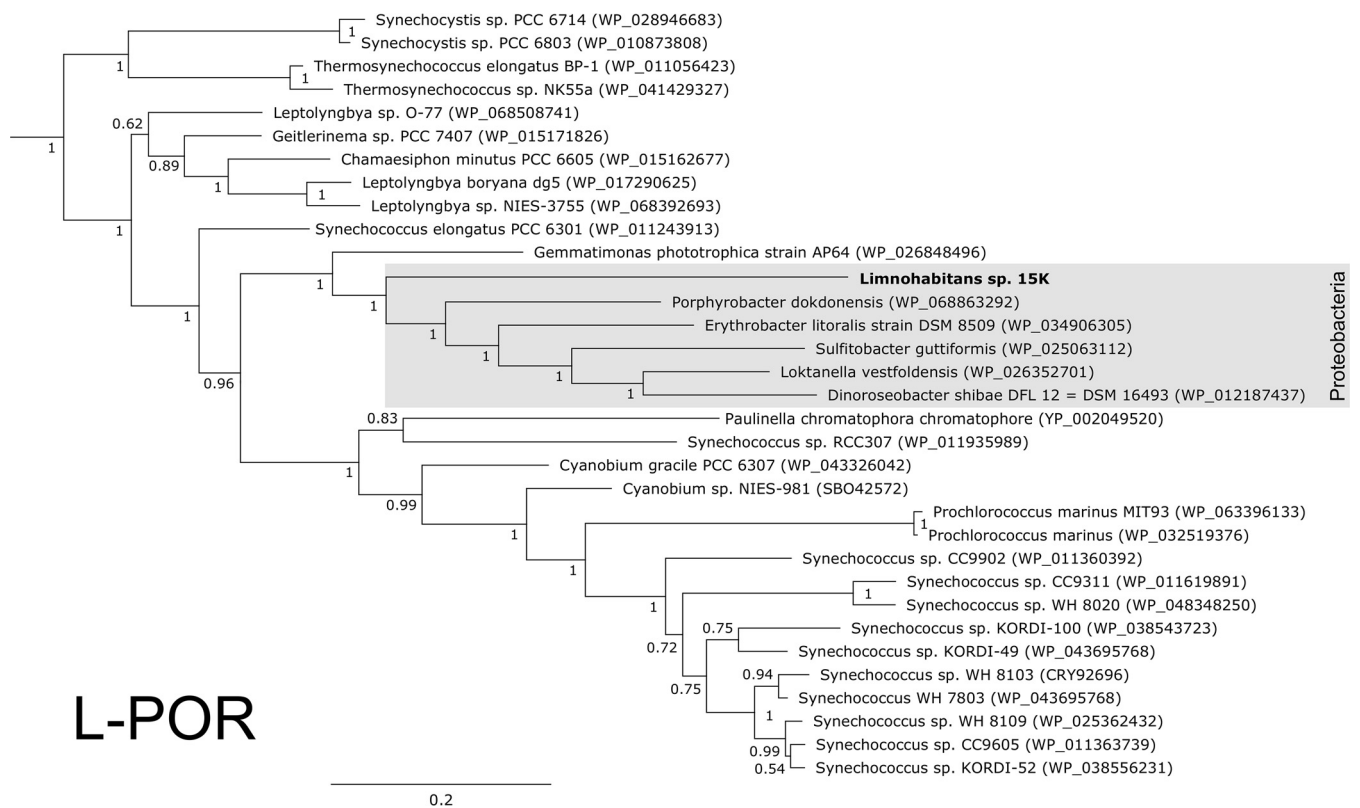


FIG 4 Phylogeny of the light-dependent protochlorophyllide reductase genes. Newly obtained sequences from two *Limnohabitans* genomes (in bold) clustered together with other proteobacterial L-POR sequences. The tree was calculated from an amino acid alignment using Bayesian inference, with posterior bootstrap support.

photoheterotrophic bacteria *Dinoroseobacter shibae*, *Erythrobacter litoralis*, and *Gemmatimonas phototrophica* and more distantly with the cyanobacteria *Synechococcus* sp. and *Cyanobium* sp. (Fig. 4).

Bacteriochlorophyll evidence in *Limnohabitans*. The expression of photosynthetic pigments was examined in batch cultures under different nutrient regimens without any detectable signal, except for *L. planktonicus* strain II-D5^T grown in YST2 medium. The sedimented phototrophic cells of *L. planktonicus* II-D5^T were pink (Fig. 5B). The recorded *in vivo* absorption spectrum documented the presence of three near-infrared (IR) peaks, at 799 nm, 813 nm, and 865 nm (Fig. 5C), originating from outer and inner light-harvesting complexes. High-performance liquid chromatography (HPLC) analysis revealed the presence of bacteriochlorophyll *a* (Bchl-*a*), hydroxyspheroidenone, and spheroidenone as the main carotenoids (Fig. 5D), which agrees with the prediction from the genomic information (*crtA* gene). Moreover, the expression of photosynthetic complexes of this strain was verified at the single-cell level using the optimized FISH protocol, which preserves Bchl-*a* autofluorescence in the near-infrared spectrum. Bchl-*a* autofluorescence was detectable at NaCl concentrations in the washing buffer below 135 mM (Table S2). This corresponds to the 20% formamide concentration in the hybridization buffer, and most probes, including R-BT065 targeting the B, C, D, and E lineages of the *Limnohabitans* genus, require much higher formamide concentrations (32). Experiments combining a high formamide concentration in the hybridization buffer with a high NaCl concentration in the washing buffer or a low formamide concentration in the hybridization buffer with a low NaCl concentration in the washing buffer (both not used in real FISH protocols) pointed to detrimental effects of high NaCl concentrations in the washing buffer. The new FISH-IR protocol allows determination of the phylogenetic affiliation of Bchl-*a*-containing bacteria. With this assay, we verified the purity of the cultures and confirmed that the cells showing

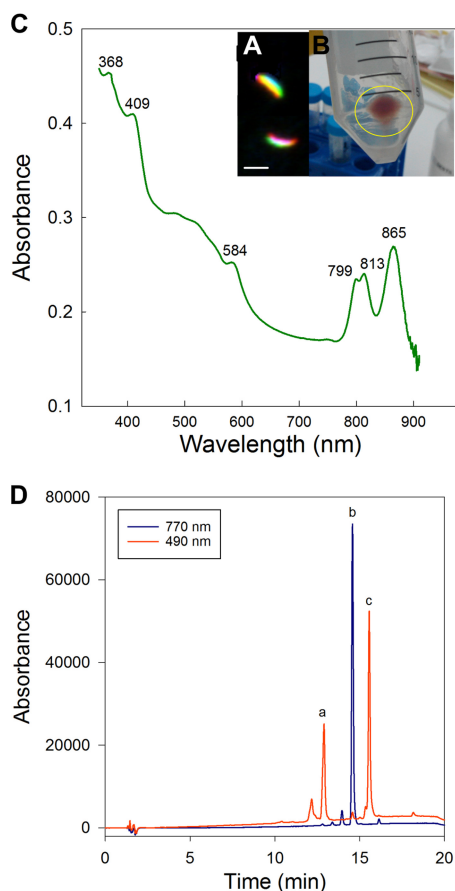


FIG 5 Pigment analysis results for *L. planktonicus* II-D5^T cells. (A) False-color photomicrographs of *L. planktonicus* II-D5^T cells hybridized with the R-BT065 probe (green), showing bacteriochlorophyll *a* autofluorescence (red). The cells were hybridized using a probe for double labeling of oligonucleotides with a Cy-3 fluorochrome, but the signal is shown green (not orange) for clarity. Scale bar, 2 μ m. (B) Pink coloration of *L. planktonicus* II-D5^T cells. (C) *In vivo* absorption of the pigmented *L. planktonicus* II-D5^T strain. (D) HPLC chromatograms of pigments extracted from *L. planktonicus* II-D5^T. Peak a (at 490 nm) corresponds to hydroxyspheroidenone, peak b (at 770 nm) to bacteriochlorophyll *a*, and peak c (at 490 nm) to hydroxyspheroidenone.

bacteriochlorophyll autofluorescence in the near-infrared spectrum were indeed *Limnohabitans* (Fig. 5A).

DISCUSSION

***Limnohabitans*, new aerobic anoxygenic photoheterotrophs in freshwater.** We showed that the genus *Limnohabitans*, in addition to chemoheterotrophic strains, contains photoheterotrophic members. We found, by sequencing of two characteristic photosynthesis genes and an analysis of potentially photosynthetic genomes, that *Limnohabitans* strains possess the characteristics of AAPs, i.e., Bchl-*a*-containing reaction centers (33) and the presence of only the aerobic form of the oxidative cyclase (*ascF* gene). Photoheterotrophic *Limnohabitans* strains, like marine *Alphaproteobacteria* strains (34), have genes organized in a photosynthesis gene cluster, including the genes for spheroidene biosynthesis (35). We demonstrated that *Limnohabitans planktonicus* II-D5^T produced bacteriochlorophyll *a* (Fig. 5); however, the synthesis of bacteriochlorophylls or carotenoids by *Limnohabitans* seems to be strongly downregulated under laboratory conditions. It is plausible that expression of the genes in the PGC can be regulated according to environmental conditions. We speculate that photoheterotrophy, which is very important in marine systems (33, 36), might be subjected to stronger regulations in freshwater systems, e.g., by the temperature (7, 8, 37). Further investi-

gations into the regulation of sunlight energy utilization by *Limnohabitans* are needed and should be facilitated by the application of our optimized FISH-IR protocol.

Environmental significance for photoheterotrophic *Limnohabitans*. Our phylogenetic analyses showed that a substantial part of the environmental betaproteobacterial *pufM* sequences retrieved from the epilimnia of freshwater lakes and rivers (15–18, 37) originated from *Limnohabitans* species (Fig. 2); for Lake Stechlin, Lake Stolp, and Lake Fuchskuhle northeast basin, they constituted 70 to 90% of all retrieved *pufM* clones (17). Moreover, several uncultured *pufM* clones and fosmids from the estuarine sites also were affiliated with *Limnohabitans* (4, 38). This is in concordance with the distribution of the R-BT cluster of *Limnohabitans* in a wide range of environmental conditions, including brackish waters (4, 20, 39). We postulate that it is photoheterotrophy combined with a broad range of carbon sources (21, 40) that give a significant advantage to the *Limnohabitans* genus over chemoheterotrophic bacteria and allows them to be regularly present within bacterioplanktonic communities, where they play a key role in the microbial food web (23). Still, the quantitative aspects of the occurrence of phototrophic *Limnohabitans* remain unknown. Here, we successfully combined two epifluorescence-based methods, i.e., FISH and IR microscopy (Fig. 5; also see Table S2 in the supplemental material). The newly developed approach should be easily applicable to environmental samples to advance our understanding of the ecology of phototrophic *Limnohabitans* and other AAPs.

Evolution of phototrophy in Betaproteobacteria. The information on bacterial photosynthesis in *Betaproteobacteria* is increasing with every new genome-sequenced genus. *Rubrivivax*, *Roseateles*, *Rhodofera*, *Rhodocyclus*, and *Methyloversatilis* were the only known genera within *Betaproteobacteria* for a long time (17, 37). Currently, other genera, including freshwater *Polynucleobacter* (41) and the genus *Limnohabitans*, have extended this list. Our data suggest that the inheritance of phototrophy in *Betaproteobacteria* was most likely vertical despite the large phylogenetic distances among the photosynthetic genera (Fig. 1). Surprisingly, most *Betaproteobacteria* members share very similar features of their PGCs, i.e., they are allocated on chromosomes, they have highly conserved gene orders, and phylogenetic analyses of the *pufM* and *bchY* genes indicated a common ancestry of these genes in *Betaproteobacteria* (Fig. 2), as was shown for the *ascF* gene (42). However, different PGC organizations were found in *Limnohabitans* sp. strains 15K and B9-3 (Fig. 3), suggesting that the PGCs in these strains had been acquired through horizontal gene transfer. Our hypothesis could be supported by (i) phylogenetic separation of their *pufM* and *bchY* genes (Fig. 2) and (ii) the presence of L-POR in strain 15K. This light-dependent protochlorophyllide oxidoreductase has long been thought to be specific to cyanobacteria, algae, and plants, while aerobic anoxygenic phototrophic bacteria usually employ the light-independent form. Recently, L-POR was confirmed also in the genomes of *Gemmatimonas phototrophica* and *Alphaproteobacteria* members such as *Dinoroseobacter shibae* and *Erythrobacter litoralis* (43).

Not all *Limnohabitans* strains have the potential for phototrophy, and many phototrophic strains are most closely related to a chemoheterotrophic bacterium (Fig. 1). This resembles the situation in the *Roseobacter* clade (44), where multiple regressive gene losses were suggested to explain the distribution of phototrophic genes (45). We suppose that such events, rather than multiple horizontal gene transfers, would predominantly explain the observed patchy distribution of phototrophy within *Limnohabitans* bacteria.

***Limnohabitans*, the freshwater *Roseobacter*?** Freshwater and marine aquatic habitats differ greatly in nutrient dynamics and are inhabited by phylogenetically distinct lineages of microbes (46), although they seem to provide similar ecological constraints, particularly the organic carbon supply and the predation pressure by flagellates. The metabolic traits and ecophysiology of the investigated freshwater genus *Limnohabitans* (21) resemble those suggested for the marine *Roseobacter* clade (47). Both groups are actively growing organisms (48, 49) under high grazing pressure (50, 51). Both clades

TABLE 2 Primer pairs used within this study

Gene(s)	Forward primer		Reverse primer		Annealing temperature (°C) ^a	PCR product length (bp)	Reference(s)
	Name	Sequence (5' to 3')	Name	Sequence (5' to 3')			
Photosynthetic reaction center (<i>pufL</i> and <i>pufM</i>)	pufL-64F	CTB TTC GAY TTC TGG RTS GG	pufM-754R	CCA TSG TCC AGC GCC AGA A	55	1,600	13
	pufL	CTK TTC GAC TTC TGG GTS GG	pufM_uniR	CCA TSG TCC AGC GCC AGA A	60	1,600	17
	pufL-4F	GCC ATG CTG ARY TTT GAR AAA	pufM-1788R	CTT GAT GGC CCA SAG GTA	50–61 (52)	1,800	This study
Bacteriochlorophyll reductase (<i>bchY</i>)	bchY_fwd	CCN CAR WSN ATG TGY CCN GCN TTY	bchZ_rev2	ART ABC CSC CNG CNC KRT CRW GRT	52	1,500	30, 66
	bchY-22F	ACA CGC ACC ATC CCG ATT	bchY-1562R	CAT GWD CCC ATC YCC TCV GMT TT	50–61 (52)	1,500	This study

^aThe annealing temperature used for PCR is indicated in parentheses.

represent generalist species with larger genomes encoding multiple enzymatic activities (including phototrophy), allowing rapid growth. Other similarities between the two groups include the chessboard distribution of photosynthesis genes (44) and the poor expression of bacteriochlorophylls under laboratory conditions in some strains (12). Both groups are frequently found in algal cultures (52–54), which suggests a specific association between algae and bacteria. This life strategy may also explain the presence of the typically “algal” gene L-POR in *Limnohabitans* strains B9-3 and 15K. Interestingly, the presence of L-POR in *Dinoroseobacter shibae* (a *Roseobacter* clade member) has also been reported, but in that organism the gene is located on a plasmid (43). In light of the current data, we propose that the highly flexible and diverse genus of *Limnohabitans* represent a freshwater counterpart of the well-known marine *Roseobacter* clade.

MATERIALS AND METHODS

Bacterial strains, cultivation, and DNA extraction. We used 46 *Limnohabitans* strains from our culture collection, including 8 novel strains (*Limnohabitans* sp. strains 2KL-51, DM1, JirI-29, JirI-31, Jir72, Jir75, Titi28, and Titi49). These strains were isolated, using the previously described procedure (21), from the Klíčava Reservoir (Czech Republic) sampled in 2008 (strain 2KL-51), from a *Daphnia magna* culture (University of Konstanz collection) sampled in 2009 (strain DM1), from the Jiřícká Reservoir (Czech Republic) sampled in 2014 (strains JirI-29, JirI-31, Jir72, and Jir75), and from a sample collected at Lake Titicaca (Peru) during the summer of 2010 (strains Titi28 and Titi49). DNA was extracted by the phenol-chloroform-isoamyl alcohol method or using the UltraClean isolation kit (MoBio, Laboratories, Inc.).

Primer design and PCR product sequencing. At least two PCRs, with the primer pairs targeting (i) photosynthetic reaction center L and M subunit (*pufL* and *pufM*) and (ii) bacteriochlorophyll reductase Y subunit (*bchY*) genes, were performed for each *Limnohabitans* strain (17, 30). The successfully amplified genes were subsequently sequenced to confirm their identity. We also designed new primers sets in order to obtain full-length sequences of the genes (for details, see Table 2). The optimal annealing temperatures for the new primer sets were determined by temperature gradient analysis (range of 49°C to 64°C, with steps of approximately 1°C). PCR products were sequenced by the Eurofins MWG Operon service.

Genome sequencing and assembly. *Limnohabitans* sp. strains JirI-31, B9-3, 15K, and G3-2 were sequenced using Illumina MiSeq v3 technology in the paired-end read module (2 by 300 bp). Reads were grouped by their names, and the low-quality regions (ends) were trimmed using the default settings in Geneious R8 (Biomatters Ltd.). Paired reads were assembled into contigs or scaffolds by using the Velvet assembler (55), the Spades assembler (56), or the assembler in Geneious R8. The assemblies obtained were compared with MUMmer 3.0 software (57) to improve the scaffolding. After genome annotation, additional scaffolding was performed manually based on conservative gene clustering. The reads were mapped onto the established scaffolds again to ensure that the scaffolding was correct.

The genome of *Limnohabitans planktonicus* II-D5^T was sequenced by Beckman Coulter Genomics (France). Two libraries were constructed, i.e., a mate pair for 454 sequencing using GS FLX Titanium technology and a fragment library for Illumina GALL sequencing (100 bp). Subsequently, 331,000 Roche next-generation sequencing (NGS) reads (110.8 Mb) and 2,000,000 Illumina NGS reads (200 Mb) were produced, resulting in about 50-fold coverage of the genome. Reads were assembled with MIRA software in two modes, i.e., (i) both 454 and Illumina reads were used for elongation of contigs or (ii) Illumina reads were used mainly for polishing of the contigs established from 454 reads. Independent assembly was performed using Newbler software, with 454 reads being used for creation and elongation of contigs and

Illumina reads being used for polishing. The assemblies obtained were manually curated and compared as described for the previous four genomes.

Genome annotation. Draft genomes were annotated using the NCBI Prokaryotic Genome Annotation Pipeline (released in 2013) (http://www.ncbi.nlm.nih.gov/genome/annotation_prok) and using the SEED and Rapid Annotation using Subsystems Technology (RAST) systems (58). In addition to newly sequenced genomes, we included *Limnohabitans* sp. strains Rim28 and Rim47 (59) in further analyses. The automatic annotation of photosynthesis gene clusters (PGCs) obtained with the RAST algorithm was manually controlled using the known PGCs of *Rhodofera fermentans*, *Rubrivivax gelatinosus*, and *Rhodobacter sphaeroides* and the environmental fosmid DelRiverFos06H03, obtained from the GenBank database. Moreover, we compared the annotations with a previous description of PGCs (60). In cases of incorrect automatic annotation, the genes were reanalyzed with the BLASTp or tBLASTx module within BLAST (<http://blast.ncbi.nlm.nih.gov>) and were reannotated using nonredundant Ref_Seq databases or a local database created from the correctly annotated strains. Similarly, nonrelated genes within the PGCs were analyzed and compared with each other to determine their conservative emergence in the *Limnohabitans* genomes.

Phylogenetic analyses. Nucleotide sequences of *pufLM* or *bchY* genes from the *Limnohabitans* strains were compared with the NCBI nucleotide database (BLASTN) and translated nucleotide sequences with the translated nucleotide database (tBLASTx). Translated nucleotide sequences were aligned using the MUSCLE algorithm (61). Three independent algorithms (neighbor joining, maximum likelihood, and Bayesian inference) were used to determine the most appropriate phylogeny, based on the amino acid composition, using Geneious R8, PhyML 3.1 (62), and MrBayes (63) software, respectively. Phylogenetic trees were visualized with FigTree v1.4.1 (Andrew Rambaut, University of Edinburgh) (<http://tree.bio.ed.ac.uk>).

Pigment determination of *L. planktonicus*, microscopy, and optical measurements. *L. planktonicus* strain II-D5^T was grown in YST2 medium (0.5 g liter⁻¹ BD Bacto yeast extract, 0.5 g liter⁻¹ BD Bacto casein acids, 0.5 g liter⁻¹ potato flour [pH 7.2]) on an orbital shaker (150 rpm) at 24°C, with a 12-h/12-h dark/light cycle (Osram Dulux L 55W/865 light source). The presence of Bchl-*a* in the culture was analyzed by HPLC. Cells were harvested by centrifugation at 6,000 × *g* for 16 min (Sigma 2-16KL centrifuge), and the pellet was resuspended in 20 μl of deionized water. Pigments were extracted with 1 ml of acetone/methanol (7:2 [vol/vol]). The clear extract was analyzed with a Prominence-i HPLC system (LC-2030C; Shimadzu, Japan) equipped with a UV-visible diode array detector, as described previously (64). Carotenoids were detected at 490 nm and bacteriochlorophyll *a* at 770 nm. Pigments were identified based on their retention times and absorption spectra.

Bchl-*a* in individual bacterial cells was detected using infrared epifluorescence microscopy (65). Cells either were fixed in formaldehyde (final concentration of 2%) or remained unfixed for immediate analysis. The purity of the cultures was confirmed by using the catalyzed reporter deposition (CARD)-fluorescence *in situ* hybridization (FISH) method (32) with the R-BT065 probe (22) and by sequencing the *pufM* gene (13).

Optimization of fluorescence *in situ* hybridization for detection of Bchl-*a* in specific phylotypes at the single-cell level. To prove that the observed autofluorescence in the infrared light came from *L. planktonicus*, we modified the FISH technique (32) to allow simultaneous detection of infrared and probe signals. The samples were fixed with fresh paraformaldehyde solution (final concentration of 2% [pH 7.5]) for 1 h at room temperature (about 21°C) or overnight at 4°C, filtered onto Nuclepore polycarbonate filters (pore size, 0.2 μm; Whatman, UK), and stored at -20°C. We decreased the hybridization temperature to 35°C and the washing temperature to 37°C, and we shortened the washing time to 20 min. The final rinsing of filters was performed in sterile deionized water and never in ethanol. Hybridized filter sections were mounted in Vectashield mounting medium.

We tested the effects of the hybridization conditions on Bchl-*a* autofluorescence with nine bacterial species, namely, *Rubrivivax gelatinosus*, *Rhodofera antarcticus*, *Rhodofera fermentans*, *Rhodospirillum rubrum*, *Rhodopseudomonas palustris*, *Methylobacter* sp., *Rhodobacter sphaeroides*, *Limnohabitans planktonicus*, and *Sphingomonas* sp. We performed triplicate tests with formamide concentrations ranging from 0 to 70%, with steps of 10%. Additionally, we combined high formamide concentrations in the hybridization buffer with high NaCl concentrations in the washing buffer and low formamide concentrations in the hybridization buffer with low NaCl concentrations in the washing buffer to identify which step is critical for successful Bchl-*a* detection upon FISH.

Upon optimization of the FISH-IR protocol, Bchl-*a* autofluorescence in *L. planktonicus* strain II-D5^T was confirmed with the R-BT065 probe (with 55% formamide in the hybridization buffer) double-labeled with Cy3 dye (Biomers, Germany). Microscopic observation was performed using an AxioVision.Z2 microscope (Zeiss, Germany) with UV and blue excitation and emission, respectively, for 4',6-diamidino-2-phenylindole (DAPI) staining, blue and green for CARD-FISH signals, green and orange for FISH signals, and white and infrared for Bchl-*a* autofluorescence.

Accession number(s). The 5 genome-investigated strains were deposited in the Leibniz Institut-Deutsche Sammlung von Mikroorganismen und Zellkulturen, and their genome sequences are available as whole-genome shotgun projects at GenBank/ENA/DBJ, with the following accession numbers: *L. planktonicus* strain II-D5^T (DSM 21594), [LFYT00000000](https://doi.org/10.1093/nar/nkz000); *Limnohabitans* sp. strain Jirll-31 (DSM 104470), [NESA00000000](https://doi.org/10.1093/nar/nkz000); *Limnohabitans* sp. strain G3-2 (DSM 104469), [NESG00000000](https://doi.org/10.1093/nar/nkz000); *Limnohabitans* sp. strain B9-3 (DSM 104471), [NESI00000000](https://doi.org/10.1093/nar/nkz000); and *Limnohabitans* sp. strain 15K (DSM 104472), [NESM00000000](https://doi.org/10.1093/nar/nkz000). Novel gene sequences have the following GenBank accession numbers: *pufLM*, [KM659099](https://doi.org/10.1093/nar/nkz000) to [KM659120](https://doi.org/10.1093/nar/nkz000); *bchY*, [KT948757](https://doi.org/10.1093/nar/nkz000) to [KT948764](https://doi.org/10.1093/nar/nkz000); and 16S rRNA genes with internal transcribed spacer, [KT899705](https://doi.org/10.1093/nar/nkz000) to [KT899709](https://doi.org/10.1093/nar/nkz000).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.02116-17>.

SUPPLEMENTAL FILE 1, XLSX file, 0.1 MB.

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