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De novo synthesis and functional analysis of the phosphatase-encoding gene *acI-B* of uncultured Actinobacteria from Lake Stechlin (NE Germany)

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Summary. The National Center for Biotechnology Information [http://www.ncbi.nlm.nih.gov/guide/taxonomy/] database enlists more than 15,500 bacterial species. But this also includes a plethora of uncultured bacterial representations. Owing to their metabolism, they directly influence biogeochemical cycles, which underscores the the important status of bacteria on our planet. To study the function of a gene from an uncultured bacterium, we have undertaken a *de novo* gene synthesis approach. Actinobacteria of the acI-B subcluster are important but yet uncultured members of the bacterioplankton in temperate lakes of the northern hemisphere such as oligotrophic Lake Stechlin (NE Germany). This lake is relatively poor in phosphate (P) and harbors on average ~1.3 x 10^6 bacterial cells/ml, whereby Actinobacteria of the ac-I lineage can contribute to almost half of the entire bacterial community depending on seasonal variability. Single cell genome analysis of *Actinobacterium* SCGC AB141-P03, a member of the acI-B tribe in Lake Stechlin has revealed several phosphate-metabolizing genes. The genome of acI-B Actinobacteria indicates potential to degrade polyphosphate compound. To test for this genetic potential, we targeted the *exoP*-annotated gene potentially encoding polyphosphatase and synthesized it artificially to examine its biochemical role. Heterologous overexpression of the gene in *Escherichia coli* and protein purification revealed phosphatase activity. Comparative genome analysis suggested that homologs of this gene should be also present in other Actinobacteria of the acI lineages. This strategic retention of specialized genes in their genome provides a metabolic advantage over other members of the aquatic food web in a P-limited ecosystem. [**Int Microbiol** 2016; 19(1):39-47]

Keywords: acI-B in Actinobacteria · phosphatases · single cell genomics · phosphate limitation · Lake Stechlin, NE Germany

Introduction

Only 1% of all bacteria on Earth are readily cultivated and there is information on ca. 60 estimated bacterial phyla [46]. Of those, at least 31 bacterial phyla have no cultured representation [17]. These bacteria have been detected in diverse habitats including aquatic and terrestrial environments [34]. In particular, lake ecosystems consist of a diverse and greatly uncultured microbiota [1,2,27]. In planktonic food webs, heterotrophic bacteria play an important role in pelagic energy flow and nutrient cycling within the microbial loop and thereby compete with phytoplankton for inorganic and organic nutrition sources such as phosphorus (P) [4,9]. Culture-independent approaches have revealed a lot of *in*

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silico information on microbial genome, but the function of any given gene cannot be determined without testing for it experimentally. Therefore, we chose an uncultured freshwater Actinobacteria, since they occur in diverse habitats and are globally distributed, making them one of the most successful bacterial phyla world-wide [47]. This universal group also dominates the microbial community in oligotrophic, temperate, and P-limited Lake Stechlin [30] in northeastern Germany [1]. 16S rRNA-gene sequence analyses and bacterial cell counts via CARD-FISH (catalyzed reporter deposition fluorescence in situ hybridization) of epilimnic bacteria, it was revealed that up to 50% of the bacterioplankton in this lake consists of uncultured Actinobacteria of the acI-B and acI-A sub-clusters [1,3].

Phosphorous dynamics in freshwaters have been well studied and it has been demonstrated that this is the most important limiting element for phytoplankton in oligotrophic freshwater ecosystems, a fact that has been termed as "the phosphorus limitation paradigm" [37,39]. Phosphorous is also considered to be the element with the lowest supply and demand ratio in many oligotrophic freshwater ecosystems [21]. Note that 80% of the whole P pool in such habitats is consumed by the bacterioplankton and solely 20% of the P pool remains left for other food web members, e.g., algae [20,22,44]. Consequently, P uptake in the epilimnion of oligotrophic lakes is to a large extent linked to bacterial communities and their remineralization via death and protozoan grazing provides substantial amounts of P to the phytoplankton [9,44]. Substrate preferences of heterotrophic bacteria, however, are largely dependent upon their genetic and metabolic potentials [11,33]. Therefore, in order to get a deeper insight into the P utilization capabilities of the dominant but yet uncultured freshwater Actinobacteria in a P-limited ecosystem, we explored the 'single-cell genome' of *Actinobacterium* SCGC AB141-P03 (representing an abundant, uncultured phylotype within the acI-B clade in Lake Stechlin). Single-cell genomics approach requires isolation of unculturable bacteria by fluorescence-activated cell sorting (FACS) followed with whole-genome amplification to conduct genome analysis at single-cell level [12]. Based on the presence of several phosphate-metabolizing genes in the genome we hypothesized that Actinobacteria of the acI-B clade should be superior in P uptake. To test the function of one of the P-utilizing genes and predict its beneficial role to the host bacterium in a P-poor environment, we overexpressed, purified and functionally characterized the putative phosphatase-encoding gene (*exoP*). To our knowledge, this is the first time, that a *de novo* gene synthesis approach in conjunction with single cell genomics has been used to study how bacteria can adapt and thrive in a P-limited environment and thereby may interfere with primary producers.

Materials and methods

Location and sampling. Lake Stechlin is a well-studied lake located in the Mecklenburg Lake District region, which originated after the latest ice age (Weichselian Stage) ca. 12,000 years ago. Oligotrophic Lake Stechlin has a maximum depth of 69.5 m and generally has high hypolimnetic oxygen concentrations, i.e., up to 60% O_2 saturation [24]. Lake Stechlin was sampled monthly from 2003 to 2015 by collecting water at various depths, except during periods of unstable ice coverage. Here, we only show 5-m depth data since the epilimnic zone is usually well mixed. In addition, the single amplified genome (SAG) *Actinobacterium* SCGC AB141-P03 of the clade acI-B was obtained from this depth. To generate a SAG library from this sample, 1 ml sample aliquots were amended with 6% (final concentration) betaine and stored at –80 °C. The SAGs were generated and identified at the Bigelow Laboratory Single Cell Genomics Center (https://scgc.bigelow.org) following previously described procedures [40]. Limnetic factors such as temperature, oxygen and pH were measured by electrodes (WTW, Weilheim, Germany). While unfiltered water samples were used for determining total phosphorus (TP), soluble reactive phosphorus (SRP) was measured after filtration of samples through 0.45-µm cellulose acetate membrane filters (Sartorius AG, Göttingen, Germany). Samples were incubated with 5% (w/v) $K_2S_2O_8$ for 30 min at a constant temperature of 134 °C in a steam autoclave. Phosphorus concentrations were then analyzed photometrically (FIA compact analyzer MLE, Dresden, Germany) following the molybdenum-blue method [23].

Phylogenetic relatedness. We conducted a phylogenetic analysis of exoP sequences predicted from all available acI draft genomes and members of another abundant lineage of freshwater ultramicrobacteria, the LD12 Alphaproteobacteria. Protein sequences were previously generated and described elsewhere [13,49]. The draft genomes are available via Genbank and "The Integrated Microbial Genomes (IMG) system" of the Joint Genome Institute (http://www.jgi.doe.gov/) [28]. Protein sequences were retrieved from IMG and aligned using the software Geneious version 7.1.4, (Biomatters, New Zealand). Homologs from *Streptomyces sviceus* ATCC 29083 and *Candidatus* Pelagibacter ubique were used as reference sequences for acI and LD12 proteins, respectively. A total of 308 positions were used to infer a maximum likelihood phylogeny using RAxML version 8.0.9 with default settings [38], implemented at the CIPRES Science Gateway. Bootstrap analysis was conducted with 1000 resamplings.

Bacterial strain, plasmid and growth condition. *Escherichia coli* Bl21(DE3) bacterial strain and pET-28a_exoP plasmid were used in this study. The *E. coli* strain was maintained and grown on lysogeny broth (LB) medium at 37 °C [36]. The antibiotic kanamycin was added to the medium at a concentration of 25 µg/ml.

Molecular genetic techniques and protein detection. Isolation of plasmid DNA, restriction enzyme digestion, agarose gel electrophoresis, purification of DNA fragments from agarose gels, electroporation, ligation of DNA fragments and several other routine molecular methods were performed using standard protocols [36]. The 918-bp putative *exoP* gene from *Actinobacterium* SCGC AB141-P03 was *de novo* synthesized by Eurofins MWG Operon (Ebersberg, Germany) and delivered in pEX-A vector. exoP_Fwd and exoP_rev primers were used to PCR amplify

the *exoP* gene and subsequently cloned in Novagen pET-28a(+) expression vector (Merck KGaA, Darmstadt, Germany) using Nde1 at 5′-end and EcoR1 restriction site at 3′-end yielding pET-28a_exoP with N-terminal fusion to $Hisx_6$. From the plasmid preparation, 200 ng were electroporated by using BioRad GenePulser (Bio-Rad Laboratories Inc., USA) at 25 µF, 200 Ohms and 2.5 kV into the electro-competent cells of *E. coli* BL21(DE3) (New England Biolabs GmbH, Frankfurt am Main, Germany). The transformants were grown at 37 °C for 1 h in SOC broth and plated on LB agar media containing kanamycin.

Protein purification. For overexpression of pET-28a_exoP in *E. coli* BL21, one liter of *E. coli* cell culture was grown at 37 °C. When the culture reached an OD_{600} of 0.5, IPTG (isopropyl β-D-thiogalactoside) was added to the end concentration of 1 mM. The culture was grown overnight at 18 °C, and then cells were harvested by centrifugation at 6000 rpm for 10 min, washed with 1 volume of TN buffer (50 mM NaCl, 50 mM Tris-HCl [pH 7.6]), resuspended in 6 ml of disruption buffer (50 mM Tris-HCl [pH 7.6], 1 mM DTT, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol), and disrupted by sonication. Cellular debris were removed by centrifugation at 45,000 ×*g* and 4 °C for 60 min. Total protein amount in the crude extracts was determined using a Nanodrop apparatus (Thermo scientific, Wilmington, USA). Ni-NTA agarose resin was used to elute His_6-fusion ExoP protein (Qiagen, Hilden, Germany). Protein fractions were loaded equally (5µg/lane) and separated by 10% SDS-PAGE. Electrophoresis and Coomassie brilliant blue staining was conducted by standard procedures [36].

Phosphatase activity assay. Phosphatase activity was determined using the EnzChek Phosphatase Assay kit (Life Technologies GmbH, Darmstadt, Germany) according to the supplier’s recommendation. As a substrate for purified ExoP, 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) was used, and the fluorescent product was detected by BioTek Synergy 2 Microplate Reader (BioTek Instruments, Inc., Bad Friedrichshall, Germany), at excitation of 360 nm and emission at 460 nm. The protein sample was replaced by buffer in the negative control experiment.

Results

Lake data. Monthly sampling of the epilimnion (5 m) of Lake Stechlin between 2003 and 2015 showed that summer water temperatures at the sampling site reached up to 21 °C, while winter temperatures were as low as 2.5 °C (data not shown). Our routine data revealed that soluble reactive phosphorus (SRP) was low, on average ~2 µg/l. However, during the winter season, when the lake was fully mixed and often covered by an ice sheet, SRP levels increased to up to ~20 µg/l. Total phosphorus (TP) level remained in the annual range of ~13 µg/l, except for the winter season (Fig. 4A). This indicated that only ~15% of TP at 5 m depths was freely available for epilimnic organisms. According to the European Environment Agency [http://www.eea.europa.eu], mean annual total phosphorus content-based categorization of European lakes exists between class1 (TP < 0.02 mg/l) to class 6 (TP ≥ 0.50 mg/l). With our current data, Lake Stechlin would fall in class1 that is minimal TP content reported.

Bioinformatics analyses. Phylogenetic analysis obtained from amino acid sequences of putative phosphatases from several acI lineages showed that the ExoP protein from *Actinobacterium* SCGC AB141-P03 was closely associated with other ExoP proteins found in acI-B tribes. The acI SAGs had up to two open reading frames (ORF) annotated as ExoP-coding genes, and both copies are included in the dendrogram. This was also the case for the *Streptomyces sviceus* reference genome, which contains two ExoP sequences that are presumably paralogs. ExoP from the LD12 tribe clustered with the ExoP from the *Candidatus* P. ubique genome, as expected (Fig. 1).

Further examination of the *Actinobacterium* SCGC AB141-P03 and other acI genomes revealed that the two putative *exoP* genes are in the same neighborhood as other P-associated genes, e.g. phosphatase- and kinase-coding genes (Fig. 4B). Genomes of the acI lineage contain several putative but specific genes potentially involved in P metabolism, e.g., exopolyphosphatase-, serine/threonine phosphatase *prpC*, polyphosphate kinase-, phosphatase *phoE*, phosphohydrolase- and phosphate-starvation inducible protein PhoH-coding genes.

Overexpression, purification and enzyme kinetics. The *de-novo* synthesized 918-bp putative *exoP* gene from *Actinobacterium* SCGC AB141-P03 was successfully overexpressed heterologously in *E. coli*. N-terminal His6-fusion ExoP was purified and a ~34-kDa protein band was visualized on a denaturing polyacrylamide gel (Fig. 2A). The purified protein activity data were plotted in the double reciprocal format (reciprocal of increasing velocity vs. varying substrate concentrations), which was first mentioned by Lineweaver and Burk [26]. The Michaelis constant (K_m) value was determined to be 125 µM at saturated substrate concentration, by using the formula (Fig. 2B):

$$\frac{1}{V} = \frac{K_m}{V_{\max}} \times \frac{1}{[S]} + \frac{1}{V_{\max}}$$

Discussion

This study aims at gaining advantage of the *de novo* gene synthesis approach in the field of aquatic microbial ecology by determining the function of a gene in an uncultured bacterium and its potential role for the bacterium’s ecology in a P-limited environment. Therefore, a putative P-utilizing

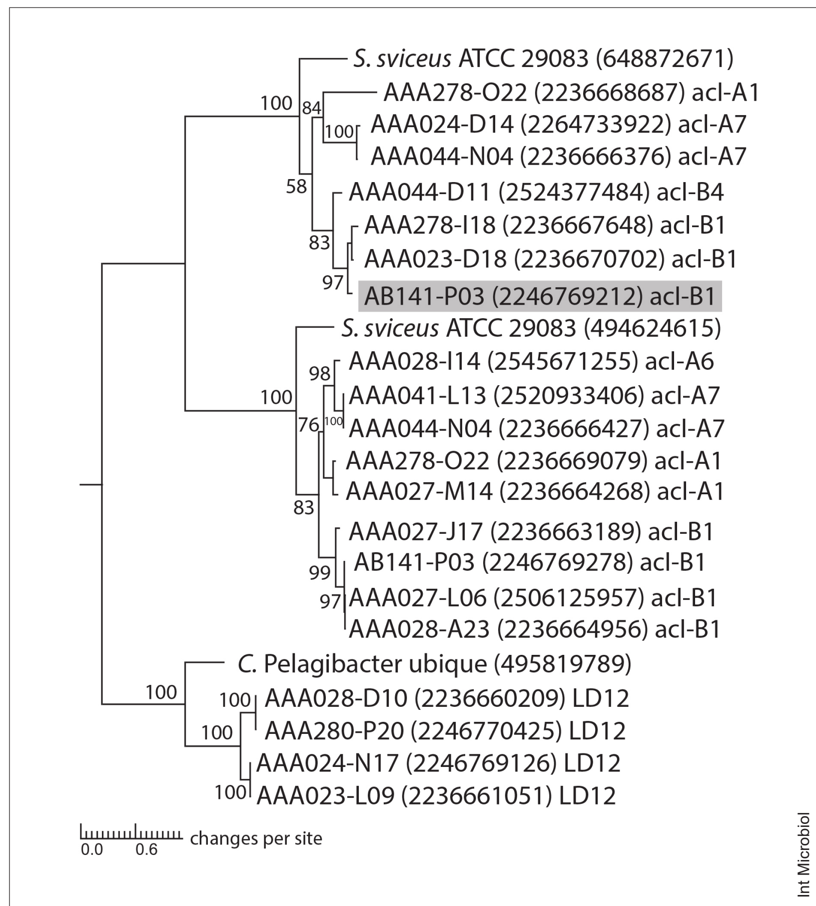

Fig. 1. Dendrogram showing the phylogenetic relatedness of amino acid sequences of putative; *exoP* genes from Actinobacteria belonging to the acI lineage and LD12 Alphaproteobacteria. Grey shade refers to the ExoP of ac1B SCGC AB141-P03 from Lake Stechlin, Germany. ExoP amino acid sequences from *Streptomyces sviceus* ATCC 29083 and *Candidatus* Pelagibacter ubique were included as reference sequences. Bootstrap values above 50% are shown next to the appropriate nodes.

gene was tested for its potential ecological role in phosphate assimilation in oligotrophic and P-poor Lake Stechlin. Uncultured A*ctinobacterium* SCGC AB141-P03 is a member of the acI-B clade within the highly abundant acI lineage of Actinobacteria in oligotrophic Lake Stechlin. Its genome reconstruction was conducted with the single cell genome sequencing approach and the genome sequence is now available at [http://www.jgi.doe.gov/] [13]. Note that *Actinobacterium* SCGC AB141-P03 has two copies of the putative *exoP* gene in its genome (estimated size of 2.0 Mbp), which indicates an important ecological function. This notion is supported by the fact that homologs of this gene are found within many acI SAGs, suggesting a key function for P uptake in a P-limited environment. Phosphate transporter subunit-, polyphosphate kinase- and alkaline phosphatase-encoding genes could be other potential candidate marker genes to explore phosphate utilization capability in unculturable acI Actinobacteria. But ABC-type phosphate transporter is composed of several subunits and therefore may increase the complexity in handling of full-reconstituted multimeric protein for functional in vitro testing with this approach. In addition, targeting polyphosphate kinase gene (*ppk1*) would answer a potential for polyphosphate granule synthesis within the cell instead of direct utilization of phosphate available in environment. An alkaline phosphatase-encoding gene is not well represented in ac1 Actinobacteria. Thus, it was tempting for us to test for functionality of the putative phosphatase protein that is not only monomeric but also present in many ac1 clades of Actinobacteria. A plethora of freshwater limnological reports demonstrate that the majority of P uptake and utilization is attributed to bacteria, but information on bacterial genes related to P metabolism, particularly of purified enzymes, and their functional role is rather limited [6,8,15,35,43]. Yet, the role of phosphatases originating from limnic microplankton, e.g., algae and bacteria, has been experimentally determined by either size fractionation experiments of natural water samples or by using various bacterial isolates. Specifically, phosphatase activities have been used as indicators for the nutrient status in a given environment or at given experimental conditions [10,45,48]. To our knowledge, this study represents a first attempt to evaluate key metabolic processes of uncultured and abundant

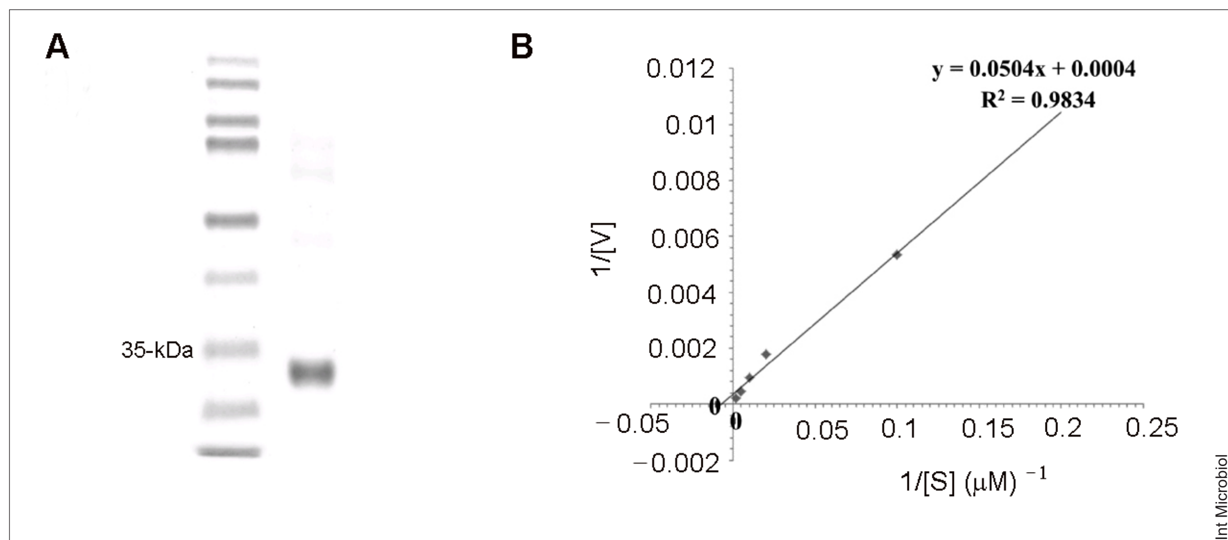

Fig. 2. (**A**) Polyacrylamide gel showing ~34-kDa purified ExoP. (**B**) Lineweaver–Burk plot representing a relationship between varying substrate concentration and reaction velocity of ExoP enzyme.

aquatic bacteria, e.g., actinobacterial phosphatase activity in environments with low P availability, by using the *de novo* gene synthesis approach.

Thereby, we demonstrate the value and feasibility of a new technical approach in microbial ecology, where a gene of interest is targeted from the information gathered from single-cell genomics, followed by *de novo* synthesis, heterologous expression and functional characterization. This represents a straightforward way of determining the function of protein-coding genes in uncultured microorganisms. The kinetic data of ExoP clearly proved that the putative enzyme ExoP was functional, suggesting that ExoP (together with other phosphatases coded from acI Actinobacteria genomes) should be central for exploiting phosphate in a P-poor environment. This suggestion is supported by the fact that phosphatase affinity (K_m value) of natural size-fractionated, particle-associated or sediment bacterial communities as well as plankton isolates ranges from 0.1 μM to ~3 mM [5,19,25,31,32,50]. Although the measured K_m value of 125 μM fits well within the range of reported K_m values, it hints to the fact that ExoP of acI-B Actinobacteria may be a weak affinity enzyme. However, heterologous expression has been performed in *E. coli* and ExoP overexpression could not be conducted in the native *Actinobacterium* to determine its true affinity. Alternatively, acI Actinobacteria in Lake Stechlin might experience situations when concentrations of SRP are low, but those of TP are high. One of these events has occurred in Lake Stechlin during the breakdown of an under ice bloom of *Aphanizomenon flos-aquae* in March 2010 [42]. Later, Bižić-Ionescu et al., have reported that large amounts of polyphosphate were released from the lysing cyanobacterial cells, and acI Actinobacteria contributed to the majority of the free-living bacterioplankton during the initial phase of bloom in Lake Stechlin [7]. During that time TP values were high, but SRP values were found to be low (see Fig. 4A) indicating that bacterioplankton with functional phosphatases have a potential growth advantage.

Several putative P-utilizing genes are widely distributed in the available acI and LD12 SAGs indicating their important role in P-sequestering of freshwater ultramicrobacteria [http://www.jgi.doe.gov/], [13,49] (Fig. 3). Exoenzyme phosphatases are capable of releasing phosphate residues from polyphosphates and a variety of biomolecules. Import of phosphorylated molecules into the bacterial cell is possible due to the presence of the partial or entire operon of genes potentially encoding phosphate translocation ABC transporters. Genes encoding phytase and pyrophosphatase (maximum up to three copies) are present in some acI SAGs, but absent in the LD12 SAGs (Fig. 3). These enzymes allow the release of soluble inorganic P, thereby providing an instant P_i repertoire for lipid metabolism and nucleic acid synthesis. Similarly, the presence of phosphonate utilization genes seems to be important for ocean-dwelling microorganisms. Several ABC transporter-coding genes potentially involved in phosphonate-P import are present in the genome of marine Actinobacteria SCGC AAA015 D07 sampled from the South Atlantic Ocean, but major genes required for phosphonate catabolism have not been found in the genome [40] (Fig. 3).

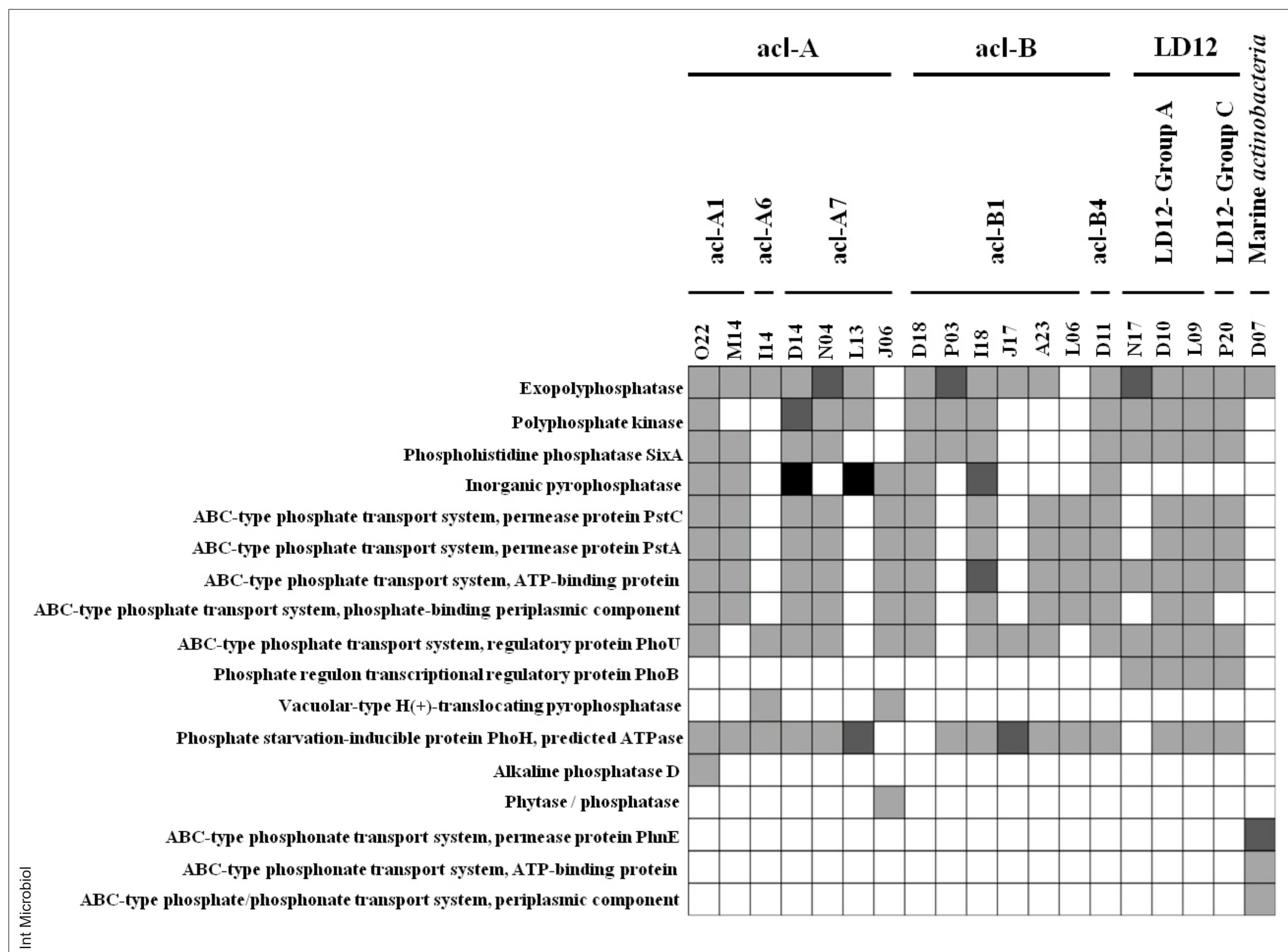

Fig. 3. Depiction of phosphate / phosphonate metabolizing protein distribution in freshwater acI and LD12 lineages in comparison to a marine *Actinobacterium*. Square symbols represent the number of occurrence of genes in the genome, i.e., not present not present (□), one (■), two (■), and three (■) Y-axis represents the important and potential proteins involved in phosphate utilization. Top X-axis represents the ultramicrobacteria harboring P-utilizing genes in the following order: acI tribes, LD12 groups and a marine Actinobacteria candidate.

Genes *exoP* and *ppk* (polyphosphate kinase) were frequently found in close proximity in acI and LD12 genomes (Fig. 4B). This could be significant for a bacterium, since phosphate concentrations can vary considerably in a limnic system. P-utilization genes in the LD12 genome were distributed in a more conserved manner than in acI genomes, which might be explained by the overall greater genetic diversity within acI lineages (Fig. 4B). The ecological importance of the *exoP* gene can be underpinned by the fact that freshwater acI Actinobacteria sometimes has two paralogs of the *exoP* gene when compared with other ultramicrobacteria such as LD12 and *Polynucleobacter necessarius* subspecies *asymbioticus* strain QLW-P1DMWA-1^{T}, which both carry only one copy of this gene (Fig. 4B), [29]. *Polynucleobacter necessarius* strains are known to be cosmopolitan and found in many diverse climatic zones and hence they have a much wider range of adaptations [16]. The frequent presence of P-utilizing genes in bacterial genomes demonstrates the great potential of bacteria to successfully compete with other organisms, e.g., primary producers, when P-availability is limited.

In conclusion, the presence of several phosphatase-coding genes in ultramicrobacterial genomes retrieved from P-limited lakes indicates a concerted focus on degradation of available inorganic and organic P sources in temporally fluctuating environments. With our current approach, we can determine the function of a protein with the help of prior annotation that indicates a potential role of a gene-of-interest. In this study, we demonstrated that the *exoP* gene indeed coded for a functional

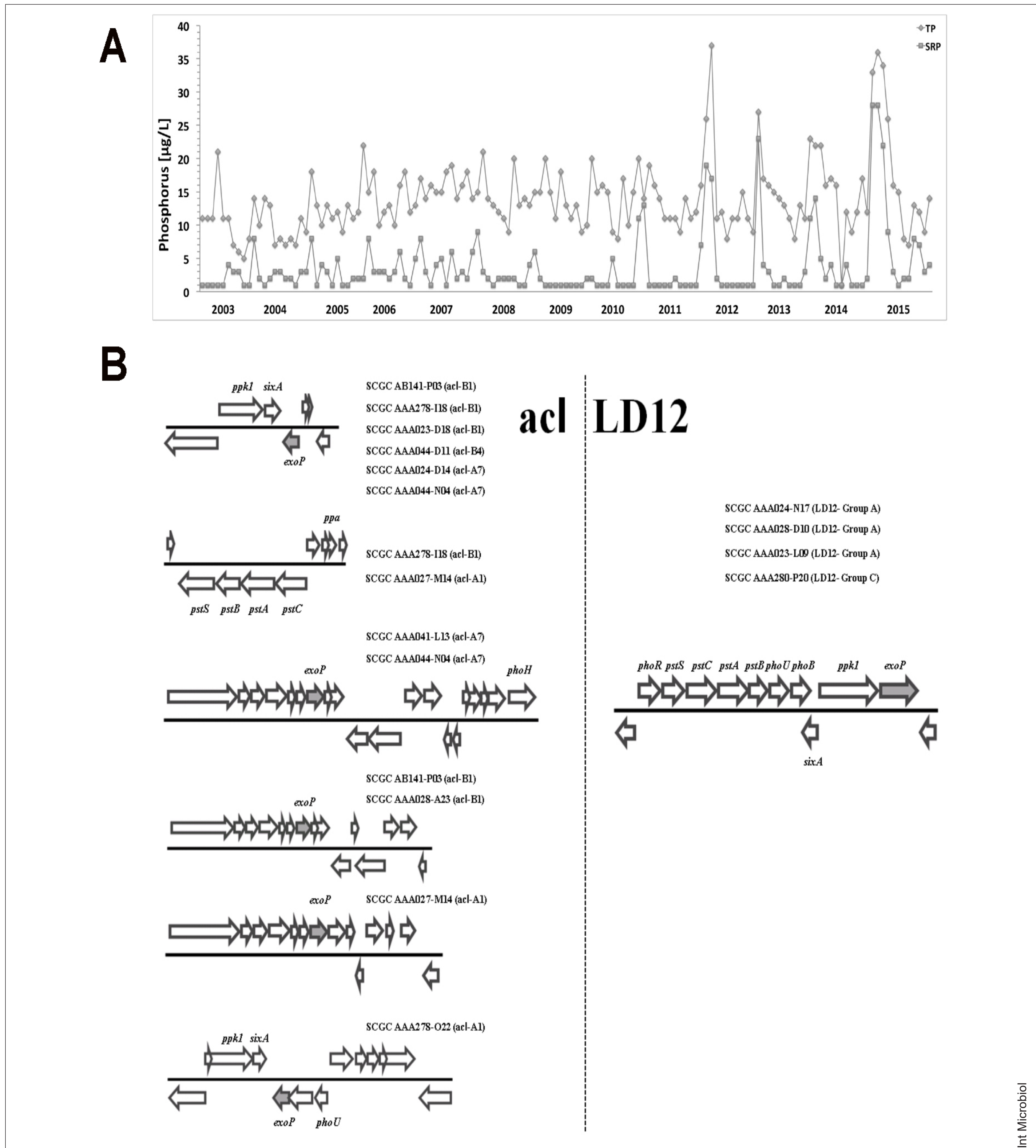

Fig. 4. (**A**) Graph representing concentrations of total phosphorus (TP) and soluble reactive phosphorus (SRP) from Lake Stechlin between 2003 and 2015. (**B**) Genetic distribution of P utilizing genes in freshwater acI Actinobacteria and LD12 Alphaproteobacteria. Gene names are abbreviated: *exoP* (putative exopolyphosphatase), *ppk1* (putative polyphosphate kinase), *sixA* (putative phosphohistidine phosphatase), *ppa* (putative inorganic pyrophosphatase), *phoB* (putative phosphate regulon transcriptional regulatory protein), *phoU* (putative ABC-type phosphate transport system, regulatory protein), *phoH* (putative phosphate starvation-inducible protein PhoH), *phoR* (putative signal transduction histidine kinase), *pstA* (putative ABC-type phosphate transport system, permease protein), *pstB* (putative ABC-type phosphate transport system, ATP-binding protein), *pstC* (putative ABC-type phosphate transport system, permease protein), and *pstS* (putative ABC-type phosphate transport system, phosphate-binding periplasmic component).

phosphatase. However, a phosphatase family might contain several members, e.g. exopolyphosphatase, pyrophosphatase, alkaline phosphatase, histidine phosphatase, lipid phosphatase etc. Whereas we did not carry out further refinement, we showed that a *de novo* gene synthesis approach can be successfully used in the field of aquatic microbial ecology. In addition to SRP, polyphosphate can substantially contribute to P sources available for bacteria in such aquatic environments, especially when SRP concentrations are low during the growing season. In Lake Stechlin, it has been shown that almost 20% of the total phosphorus from the sediment consists of polyphosphate [18], and that polyphosphate storing bacteria are abundant in the water column and sediment [14]. Hence, the presence of P-utilizing genes by ecologically successful bacteria, particularly acI Actinobacteria, indicates a valuable adaptation to a P-poor aquatic ecosystem. In order to understand the genetic potential of any uncultured microbe, *de novo* gene synthesis approach appear to be relevant to address a multitude of microbial metabolism related hypotheses in ecology. Caution, however, needs to be taken since a wrong pre-annotation of a gene might lead to an undesirable experimental outcome. Apart from annotation biases, heterologous expression in another host might result in a failure of functional protein synthesis. Nevertheless, our study demonstrates that the *de novo* gene synthesis approach can be successfully used to evaluate gene function from whole gene sequences of so far uncultivated bacteria and thereby circumvents the often labor-intensive cultivation step of environmental microbes.

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Competing interests. None declared

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