GENERALIST AND SPECIALIST STRATEGIES OF PHOSPHORUS ACQUISITION BY AQUATIC BACTERIA

By

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ABSTRACT

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Resource heterogeneity increases biological diversity by providing opportunity for niche partitioning and resource specialization. Organisms which use few of the available resource forms are considered specialists, while those which use many resource forms are considered generalists. The relative proportion of specialists and generalists within a community impacts ecosystem functions, such as total productivity. Being a resource specialist or generalist may come with a fitness cost or favor performance tradeoffs. For example, generalists may suffer a fitness cost for maintaining a broad ecological niche.

Heterotrophic microbes and primary producers have the potential to specialize on different chemical forms of essential nutrients such as nitrogen and phosphorus, yet there have been few studies of nutrient specialization, limiting our understanding of associated costs or performance tradeoffs. In the present study, we quantified phosphorus resource specialization by aquatic bacterial isolates and tested for a specialization-performance tradeoff, using bacterial growth rate as the measure of performance. We found evidence for bacterial specialization on phosphorus form and for an environment-specific specialization-growth rate tradeoff. Our results indicate that nutrient-based resource specialization can strongly influence an important performance trait of an organism, but these affects may be environment-specific. Results from this study improve our understanding of how a species' niche breadth may impact its ecological strategies and competitive outcomes.

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CHAPTER 1

INTRODUCTION

An organism's ecological niche can be described both in terms of its resource requirements as well as the way its activities influence its environment (Chase & Leibold 2003). In theory, species fill finite quantities of resource space according to their traits. Some species are considered to be ecological 'specialists,' having a narrow niche breadth and relatively stringent ecological or environmental requirements to satisfy their resource needs, while ecological 'generalists,' have a broad niche breadth with respect to the way they meet their resource requirements. Communities comprised primarily of specialists may maximize ecosystem resource usage through functional complementarity (Loreau 2001), while generalists may play important roles in maintaining ecosystem stability and functions (Richmond *et al.* 2005; Mou *et al.* 2008). Research suggests that neutral processes may dominate species distributions in some systems (Hubbell 2001), but niche partitioning cannot be ruled out as an important driver of community composition and ecosystem functions in many systems (Levine & HilleRisLambers 2009).

Not only macroorganisms, but microorganisms too can be described by their ecological niche and demonstrate niche specialization. However, microbial interactions occur at the micrometer scale and smaller, so while 'seed size' may be an ecologically relevant food preference for a bird, 'molecule structure' may be a more ecologically relevant food preference for microorganisms. For example, Upton and Nedwell

compared the abilities of oligotrophic and copiotrophic bacteria to use a suite of carbon sources for growth (Upton & Nedwell 1989). They found that oligotrophic bacteria were able to use more carbon substrates, thus demonstrating a broader niche breadth. Similarly, Mou *et al*. compared bacterial communities' carbon niche breadth in a salt marsh when supplemented with one of two carbon sources (Mou *et al*. 2008). Using DNA-based methods, they found that most bacteria in their study tended to be generalists. Microbes have also been shown to specialize on other resources such as light (Stomp *et al*. 2004).

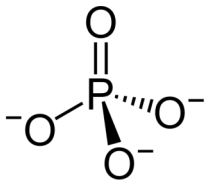
Microbial consumption and transformation of nutrient resources affect global processes, such as oceanic primary productivity, biomass transfer, and nitrogen fixation (Falkowski *et al*. 2008). As a frequently limiting nutrient in aquatic ecosystems, phosphorus (P) resources hold a key to ecosystem productivity and functions (Dyhrman *et al*. 2007). Inorganic phosphate (P_i) concentrations can be as low as <30 pM in freshwater environments and <50 nM in the ocean (Karl 2000, Bjórkman & Karl 1994, Hudson *et al*. 2000). Organic P (P_{org}) concentrations are typically much greater, since many of the compounds that comprise this pool require hydrolytic enzymes for organisms to access the P. As potentially better competitors for P_{org} than eukaryotic phytoplankton, bacteria may control the quantity of P available to eukaryotic phytoplankton and ultimately primary productivity in some ecosystems (Currie & Kalff 1984, Coveney & Wetzel 1992, Cotner & Biddanda 2002). Excess P release into surface waters promotes lake eutrophication, which can lead to toxic algal blooms, fish kills, reduction in recreational value, and decreased drinking water quality (Carpenter *et*

al. 1998). Since microbial communities are essential intermediaries in the uptake and transformation of these P resources, further research into the processes that control microbial P transformations is needed as we develop strategies for remediation of eutrophied waterbodies.

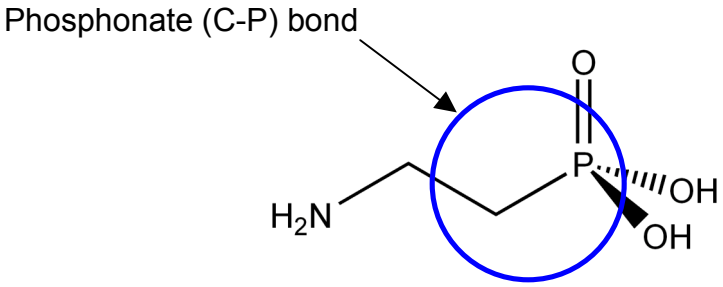
Bacteria employ many strategies to acquire P, such as expressing high- and low-affinity P-uptake proteins, and secreting and excreting phosphatases. Perhaps the most well studied mechanisms for accessing P_i are the low-affinity, constitutive Pit system and the high-affinity, P_i-repressible Pst system expressed in *Escherichia coli*. To access P from P_{org}, bacteria maintain a genetic arsenal of P-acquisition enzymes. Many of these enzymes and their encoding gene or gene clusters can be found in Table 1-1. Bacteria commonly use nonspecific acid or alkaline phosphatases, which cleave P from phosphomonoesters. These enzymes may be attached to the cell membrane, contained within the cytoplasm or periplasm, or excreted into the environment (Luo *et al*. 2009; White A. 2009). Bacteria may also utilize substrate-specific enzymes, such as phytases, which cleave phosphomonoesters from bulky phytate compounds, or phosphonatases, which cleave C-P bonds in phosphonate compounds (Table 1-1 and Figure 1-1). In addition, some bacteria are able to take up certain small molecules in their entirety, such as adenosine monophosphate (AMP), cyclic adenosine monophosphate (cAMP), and glycerophosphoric acid (White 2009 and references therein). Figure 1-1 displays several examples of P_{org} bond types and indicates enzymes that bacteria frequently use to cleave P from a variety of resource forms.

Table 1-1. Organophosphate utilization enzymes of bacteria. Shown are many common bacterial enzymes, primary genes or gene clusters which encode the enzymes, and the phosphorus resource(s) they target.

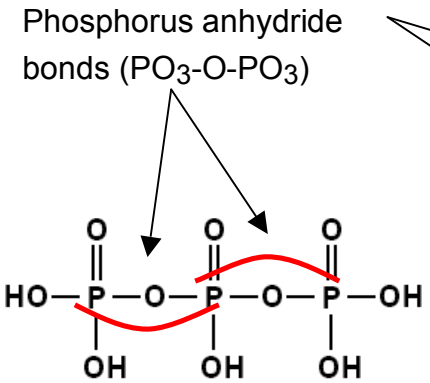
Enzyme	Encoding gene(s) or gene clusters	Primary substrate(s) targeted	References
Alkaline phosphatase	*phoA*, *phoD*, *phoX*	phosphorus esters	Luo *et al.* 2009
Acidic Phosphatase	*appA*	phosphorus esters	Vershinina & Znamenskaya 2002
Phytases	*phy*	phytate	Lim *et al*. 2007
C-P lyase	*phn* gene cluster	many phosphonates	Huang *et al*. 2005
Phosphonatase	*phnW*, *phnX*	primarily 2-aminoethylphosphonate	Huang *et al*. 2005
Polyphosphatase	*ppK*	polyphosphate	Vershinina & Znamenskaya 2002
Phosphonoacetate hydrolase	*phnA*	phosphonoacetate	Gilbert *et al*. EM 2009
5'-Nucleotidase	*nuc*	5' -nucleotides	Vershinina & Znamenskaya 2002



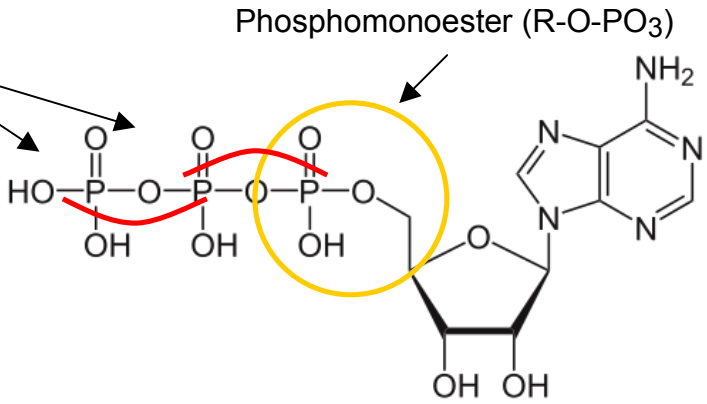
Inorganic phosphate ion



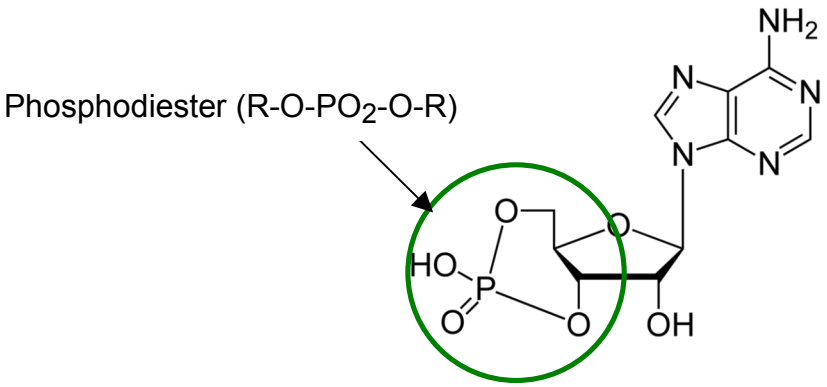
(2-aminoethyl) phosphonic acid



Short polyphosphate chain



Adenosine triphosphate



Cyclic adenosine monophosphate

Figure 1-1. Examples of organic phosphorus bonding types. Many phosphorus resources are bound up in organic forms. Phosphonates contain stable C-P bonds (circled in blue), while phosphate esters contain

Figure 1-1 (cont'd)

more labile C-O-P bonds (circled in yellow). Monoesters have one C-O-P bond, while di-esters (circled in green) or tri-esters (not shown) have two or three C-O-P bonds, respectively. Inorganic phosphate ion ("free phosphate") and a simple polyphosphate with phosphorus anhydride bonds (shown with red curves) are included for comparison. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis.

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CHAPTER 2

GENERALIST AND SPECIALIST STRATEGIES OF PHOSPHORUS ACQUISITION BY AQUATIC BACTERIA

Abstract

Resource heterogeneity provides opportunity for ecological specialization. Organisms that use few of the available resources are specialists, while those that are capable of using many resources are generalists. Theory predicts that there are costs and tradeoffs with being a specialist or generalist, the magnitude of which may depend on environmental conditions. For example, specialization is considered to be most advantageous in homogeneous environments with abundant resources, where generalists may suffer a large fitness cost for maintaining a broad ecological niche.

Although there is evidence that microorganisms have the potential to specialize on different forms of an essential nutrient (e.g., phosphorus), there have been few studies on nutrient specialization, limiting our understanding of associated ecological strategies or performance tradeoffs. In the present study, we measure bacterial growth rates, an essential fitness component, for thirty-nine bacterial strains isolated from an oligotrophic and eutrophic lake on a suite of phosphorus (P) resources. We then quantified P niche breadth and tested for a specialization-performance tradeoff. We found that bacterial isolates specialized on a diverse range of P forms, and that there was a positive linear relationship between P specialization and an isolate's maximum growth rate, but only for isolates originating from the more eutrophic lake. These results

highlight the potential for P resource heterogeneity and nutrient specialization to drive ecological strategies and performance tradeoffs in microorganisms.

Introduction

Resource heterogeneity plays a large role in driving and maintaining earth's biodiversity. Because resources are typically limited, species have evolved a variety of ecological strategies to effectively meet their nutritional and energetic needs. For example, species may evolve ecological tradeoffs that allow them to maximize access to particular resources, but at a cost; stockpile resources while they are abundant; or remain dormant until environmental conditions are more favorable (Cáceres 1997, Caley & Munday 2003, Jones & Lennon 2010). Organisms' ability to effectively compete for and acquire resources strongly impacts species distribution, community composition, and ecosystem functions.

Theory predicts that species' niche breadth, or the number of different resource forms that a species can use to meet its growth requirements, should be directly impacted by resource availability (Futuyma & Moreno 1988, Chow *et al.* 2004). Organisms frequently take advantage of resource heterogeneity by partitioning available resources. Those which use only a small number of the available resource states are considered niche specialists, while those which use many of the available resource states are considered niche generalists. The proportion of specialists to generalists in a community can impact total resource use, productivity, and the relationship between community diversity and ecosystem function (Finke & Snyder 2008, Gravel *et al*. 2011).

Even resources that seem homogeneous to us may in fact contain multiple ecologically relevant resource states for certain groups of organisms. For example, while light is typically considered to be a single resource, photosynthetic pigments only absorb photons from a portion of the spectrum, allowing for phytoplankton to partition the light spectrum and ultimately coexist (Stomp *et al*. 2004). Nutrient resources are also diverse and have been shown to be an important axis of niche variation. Essential nutrients are bound up in many chemical forms, which are more or less biologically available to different organisms. Variation in ability to access nutrient resource forms can influence species' resource partitioning, determination of species dominance, and persistence of less dominant species in communities (McKane *et al*. 2002, von Felten *et al*. 2009).

Ecological and evolutionary constraints limit species' niche breadth. Ecological constraints include organisms' physiological limitations, such as the necessary allocation of energy to different aims (i.e. fast growth, reproduction, or predator defense). Maintaining a broad ecological niche may have inherent energetic costs or favor performance tradeoffs (Futuyma & Moreno 1998). For example, traits that increase fitness in one environment may decrease it in others (Kassen 2002). Specialists are theorized to evolve in constant environments with abundant resources, while generalists should evolve in temporally variable environments with heterogeneous resources (Futuyma & Moreno 1988, Chow *et al*. 2004). So while a narrow-niche specialist may perform better than a broad-niche generalist in its preferred environment, the generalist may perform less well, but more consistently across environments (Caley & Munday 2003). However, the shape of performance tradeoffs are highly system-

specific and can vary depending on the environmental conditions (Jessup & Bohannan 2008). Perhaps for this reason, ecological and evolutionary performance tradeoffs are frequently theorized, though only occasionally empirically confirmed. Evolutionary constraints on niche breadth can include genetic incompatibilities among traits, such as occurs when there is genetic correlation between a trait subject to directional selection and one subject to antagonistic selection (Futuyma 2010). Additionally, specialized traits may evolve rarely, constraining such traits to certain phylogenetic groups. Such a trait is considered to be 'historically constrained' or 'phylogenetically conserved' (Prinzing *et al.* 2001).

Phosphorus (P) is an essential limited resource for all living organisms. It is a primary component of membranes, nucleic acids, and regulates protein snynthesis. Heterotrophic microbes and primary producers are crucial for the transformation of dissolved P resources into biomass. Inorganic phosphate (P_i) is considered to be the most readily available form of P, being easily taken up by plants and microbes without the requirement for specialized enzymes (Dyhrman *et al*. 2007). Yet the dissolved P in most ecosystems is largely bound in organic forms and requires specialized, microbially produced enzymes to be accessed. Within this pool of organic phosphorus (P_{org}), there is substantial variability in compound lability based on chemical structure. For example, phosphate esters—P_{org} compounds with C-O-P bonds—appear to be more biologically available than phosphonates, which have a more stable C-P bond, possibly because there are fewer enzymes which facilitate the breaking apart of such compounds (Clark *et al*. 1998). The opportunity to acquire P from many resource forms has been shown to

be an important driver of genetic diversification for microbes and influences microbial community composition, including species dominance in low-nutrient areas of the ocean (Frette *et al*. 2009, Zubkov *et al*. 2003, Martiny *et al.* 2006). Despite the importance of microbial P transformation in ecosystems and the demonstrated opportunity for niche-specialization, we do not know the extent of variation in microbial P-use niche breadth, and our understanding of the relative availability of different P resource forms for microbes remains limited.

Here, we isolated aquatic bacterial strains from different environments to explore variation in P niche breadth, and test for a tradeoff between growth-rate and P niche breadth. In this study, we compare bacterial isolates' ability to grow on a suite of different forms of phosphorus, chosen for their ecological relevance in aquatic environments and molecular structural diversity. We hypothesized that bacteria would vary in their P niche breadth and demonstrate a performance tradeoff, such that those with a wider niche breadth would on average grow slower than those with a narrower niche breadth in their preferred environment (or P source). We also predicted that bacterial isolates would grow at different rates on compounds with chemical structures as similar as ATP and GTP, indicating a fine level of compound recognition among P resources.

Materials and Methods

Lake Characterization

In fall of 2009, we collected surface water samples (0.5 m) from two southwest Michigan lakes near the W.K. Kellogg Biological Station, USA: Wintergreen (WG) Lake

and Little Long (LL) Lake. WG Lake is an eutrophic waterbody located within Kellogg Bird Sanctuary, and receives large P inputs from the resident birds. Little Long is an oligotrophic lake with marl clay sediments and no known point-source P loadings. Both lakes are sampled for nutrients several times each year as part of a regular monitoring program. We referenced three years of nutrient data (2007-2009) for this study.

To determine bacterial community similarity between these two lakes, we analyzed previously collected DNA pyrosequencing data (Jones & Lennon 2010). Briefly, mixed surface layer samples were collected in summer of 2008. 250mL water was filtered onto 0.2mm filters, and DNA was extracted using a commercially available kit (DNA FastPrep purification kit from BIO 101). Using PCR, the DNA was labeled with barcoded primers targeting the V4 region of the 16S rRNA gene before being sequenced with an Illumina Genome Analyzer II at the Research Technology Support Facility (RTSF) at Michigan State University. From 982 total sequences, only unique (622 total), well-aligned (482 total) sequences were included for analysis.

Only unique tag sequences from the epilimnia communities were included for analysis, reducing the total number of sequences from 982 to 622. Following sequence alignment and quality checks for correct position and size, the number of included sequences was further reduced to 496. These sequences were then binned according to 97% nucleotide similarity for analysis. Using the libshuff program within the package Mothur v1.23.1 (Schloss 2009), we calculated the Cramer-von Mises test statistic to test for bacterial community similarity between the two lakes (Singleton 2001).

Bacterial Enrichment and Isolation

We enriched for bacteria in a variety of P environments. Immediately following sample collection, we spread-plated 50-100 μL water samples from each lake onto 1.5% washed-agar plates containing a modified WC minimal medium based on Stemberger 1981 (see Appendix B for full recipe). Briefly, the medium contained a minimal nutrient and trace element mixture with the addition of the vitamins thiamine (vitamin B_1) and biotin (vitamin H), and one source of P. WC agar plates were prepared at two P concentrations (100 μg P/L and 1 mg P/L), using one of five sources of P [inorganic phosphate (P_i), (2-amino-ethyl) phosphonic acid (AEP), adenosine triphosphate (ATP), phytic acid (Phyt) or a combination of all of the compounds], and were buffered with calcium carbonate. The agar was washed by rinsing with distilled water until the rinse water remained clear, with a miminum of seven rinses. It was then rinsed once with Nanopure water, once with 70% ethanol, and finally with acetone before being aerated at 40˚ C until dry. We allowed these enrichment samples to incubate at 25˚ C for two to four weeks, to allow enough time for slow-growing bacteria to form colonies.

Strain isolation

We sought to isolate diverse lake bacteria with different P-utilization strategies, so we selected colonies for isolation that were morphologically distinct, sampling from each type of P enrichment. We isolated the bacteria on agar plates using our modified WC media (mWC) with the addition of P_i as the P source which we assumed would be readily accessible to all bacteria, 10 mM HEPES buffer, and 50 mg/L cylcohexamide to prevent fungal contamination. This recipe (mWC) was used to make all subsequent

media, with the desired P resource added. Also note that for all media, the stoichiometry of the compounds was taken into account, and each P resource was added according to the total P added, rather than the compound concentration. Isolates were re-streaked multiple times to ensure single-strain isolation, and then grown in 1mg P/L P_i mWC broth before being cryopreserved (19% glycerol, 81% 720 μg P/L P_i mWC, final concentration). We preserved 18 isolates from LL Lake and 21 from WG Lake.

P-utilization assays

We tested each isolates' ability to grow on 19 P sources, chosen for their relevance in aquatic ecosystems and diversity of P-bonding structures (see Tables 1-1 and 2-1). Assays were carried out in 96-well plates, using mWC broth containing one of each P source at a target concentration of 5 mg P/L. We maintained high P concentrations in order to ensure that the bacteria were not nutrient limited during exponential growth. Each treatment was conducted in quadruplicate, with four positive control wells containing P-free media, and 16 negative controls containing media with P_i. The negative controls were positioned along either edge of the plate to alleviate potential edge effects. Prior to initiating an assay, cryopreserved isolates were inoculated into mWC with P_i [1 mg P/L] and incubated in 10 ml of liquid medium in 125-ml shaken flasks (160 rpm) at 25˚ C until turbid, at which time they were diluted 10-fold with P-free mWC and inoculated at 10% total volume into each of the 80 treatment or positive control wells in a 96-well plate (20 μL inoculum into 180 μL media). Negative controls received 20 μL P-free media. The plates were then incubated at 25 ˚C for up to

16 days. Each wells' optical density at 600 nm was measured using a Molecular Devices SpectraMax5 spectrophotometer every 2-24 hours, depending on the speed of the life cycle of each isolate. We used maximum likelihood (ML) to fit the modified Gompertz function (Zwietering *et al.*1990; Lennon *et al.* 2007), which estimates ecologically relevant parameters, in particular lag phase, maximum growth rate, and maximum cell concentration (here, maximum optical density).

In order to account for any growth in the control wells, presumably due to stored P ('luxury growth,' Bolier *et al.* 1992) we subtracted the average value of the P-free control wells from all treatment wells for the isolate. We calculated an average growth rate from the quadruplicate wells to obtain a single growth rate value for each isolate on each P source. While, as stated previously, we used maximum likelihood to find the maximum growth rate of each isolate on each P source, we will refer to these maximum growth rates (per isolate, per P source) simply as 'growth rates' (GRs). Further, for the rest of this paper, an isolate's 'maximum growth rate' (max GR) is considered to be its maximum GR on the single P source on which it grew fastest. We standardized isolate GRs for nearly all statistical analyses by dividing each isolate's GR on each P source by its own max GR. Standardizing in this way accounts for disparate isolate GRs as an inherent property of each isolate, irrespective of the P source on which it grew. These standardized growth rates are constrained to a scale from 0-1, with any differences among isolates representing differences in their relative P-use abilities rather than absolute differences in growth rates on the different P sources. We also excluded the P source B12 from further analysis, since no isolates demonstrated detectable positive growth on it, neither per optical density at 600 nm nor visual inspection. We created a

heatmap to visually display all isolate GRs on all P sources using the heatmap.2 function within the gplots package in the R statistical environment (R Development Core Team 2004).

DNA sequencing and tree construction

We sequenced the isolates' DNA and constructed phylogenetic trees to test for the influence of phylogenetic history on P-use traits. We extracted DNA for sequencing from fresh broth cultures of isolates inoculated from cryopreservation, grown to turbidity. We used polymerase chain reaction (PCR) to amplify a portion of the encoding for a region of the16S rRNA gene using the universal bacterial primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') and the following thermal cycle conditions: 5 min at 95 ˚C (initial denaturation); 30 cycles of 1 min at 94 ˚C, 1 min at 58 ˚C, 2 min at 72 ˚C; 10 min at 72 ˚C). The amplified DNA was purified using the Qiagen Quick nucleotide fragment clean-up kit and sequenced on an ABI PRISM® 3730 Genetic Analyzer at the Research Technology Support Facility at Michigan State University. To align the sequences, we first used the quick-alignment tool provided in ARB software (http://www.biol.chemie.tu-muenchen.de), followed by manual refinement based on known secondary structures (Ludwig *et al*. 2004). We assigned each sequence a genus designation, as determined using the Classifier tool provided by the Ribosomal Database Project (RDP, Wang *et al.* 2007).

We then constructed a phylogeny from the aligned sequences with a general time-reversible model of evolution (GTR) using the software package BayesPhylogenies (Pagel & Meade 2004). Rather than returning a single consensus tree,

BayesPhylogenies uses Markov Chain Monte Carlo (MCMC) methods to generate a suite of trees, whose frequency distributions correspond to the certainty of a given tree. This is a way of incorporating phylogenetic uncertainty into subsequent analyses. We chose the GTR method after using the freely available software package jModelTest (Guindon & Gascuel 2003; Posada, D. 2008) to statistically compare many different models of evolution to determine which is best for the given data. We specified the *Bacillus subtilis* strain as the outgroup and allowed the chain to run for 2,000,000 iterations, with a burn-in period of 10,000 iterations. Thereafter, we sampled every 500 trees to yield a total of 3,981 trees.

Statistical Analyses

We assessed P niche breadth using the Levins index (Levins 1968) with the standardized GRs. The Levins index incorporates the number of resource states used (i.e. the number of P sources) as well as the relative frequency with which they are used (the standardized GR). Higher values indicate a broader niche breadth. We ran a multiple regression to explain the Levins index as a function of max GR (a continuous variable) and lake origin (a categorical variable). The max GRs were $\log_{10}$-transformed to meet the assumption of equal variance. Since we had different numbers of isolates from each lake, lake origin was an unbalanced covariate. To avoid autocorrelation of the data, we analyzed the data using type II sums of squares with the 'car' package in the R software environment (Fox & Weisberg 2011). We also determined whether lake origin significantly influenced isolate growth across all P sources by conducting an analysis of similarity (ANOSIM) using the vegan package within the R statistical environment

(Oksanen *et al.* 2011, R Development Core Team 2004). We compared results when using Bray-Curtis, Euclidean, and Manhattan distance matrices. To assess the significance of the ANOSIM statistic, we ran 10,000 permutations of the data.

The P sources used in this experiment were chosen to maximize our understanding of how bacterial P-use traits might relate to special enzymes or certain molecular structures, and to compare P-use abilities among even structurally similar compounds. In order to quantify similarity among P sources, we subjected the P-use data to cluster analysis using the R software package 'pvclust' (Suzuki & Shimodaira 2009). Multiple methods for computing distance matrices (Manhattan, Euclidean, and Bray-Curtis) and for conducting cluster analysis (Group Average and Ward's Method) were compared to ensure reliability of the results. Approximately Unbiased (AU) p-values were calculated from multiscale bootstrap resampling for 10,000 iterations. The hypothesis that "the cluster does not exist" is rejected with significance level at 0.05 for clusters with AU p-value > 0.95.

Phylogenetic influence

Finding that a trait correlates highly with a clade's phylogenetic history indicates that there is some degree of phylogenetic conservatism, i.e. the variance among extant species is explained well by ancestral relationships. Phylogenetic conservatism necessitates non-independence of the data and possibly the need for phylogenetic correction. Therefore, where phylogenetic history correlates with the trait data, we present results for both traditional analyses without phylogenetic correction and those including phylogenetic correction. To find maximum likelihood (ML) values for a given

trait for each of the 3,981 phylogenetic trees, we used the software program BayesTraits (Pagel 1997, Pagel 1999). One can test whether a phylogeny correctly predicts species trait covariances by incorporating the parameter Lambda in analyses. Likelihood ratio tests can be used to compare models in which Lambda assumes its ML value, or is forced to be 1 or 0. If the likelihood when Lambda assumes its ML value is not distinguishable from the likelihood when it is forced to be 1, then the trait has evolved as expected, given the tree topology and model of evolution. Similarly, if the likelihood when Lambda assumes its ML value is not distinguishable from the likelihood when Lambda is forced to be 0, this is evidence that the trait has evolved completely independently of the phylogeny and phylogenetic correction is unnecessary.

All such analyses are dependent upon the model of evolution used for the analysis. We used a random walk model of evolution, incorporating the scaling parameter Kappa for each trait or comparison between traits. The Kappa parameter in BayesTraits is used to stretch and compress branch lengths, allowing one to test for a gradual versus a punctuational mode of evolution and incorporate the finding into the model of evolution used. We allowed the program to first estimate the maximum likelihood value of Kappa for each trait or comparison between traits, and then incorporated the mean value to the hundredths place in our analyses. We also allowed the program to estimate the maximum likelihood value of Delta, a parameter used to scale total path length in a phylogeny, allowing one to compare models varying the relative import of earlier versus later trait changes. We found that even though this parameter was much greater than the Brownian Motion default of 1.0, indicating that later trait changes correlated better with the phylogeny, incorporating this parameter did

not significantly improve the likelihood of the evolutionary model. The Lambda parameter was incorporated in all analyses with this program, as it is the inclusion and restriction of this parameter that allows one to conduct phylogenetic conservatism hypothesis testing. All statistical analyses aside from those involving the pyrosequencing data were performed with R software (R Development Core Team 2004).

Results

Lake Characterization

Despite their proximity, LL Lake and WG Lake contrast greatly in both nutrient concentrations and microbial composition. As shown in Table 2-1, the total P content of WG Lake can be 15 times that of LL Lake. While the P_i content of LL Lake remains at or near the detection limit, the inorganic nitrogen concentrations are far more abundant than those of WG Lake. This suggests that LL Lake is likely P-limited, while WG Lake may be more nitrogen-limited. The lake contrasts are also evident when comparing the epilimnetic bacterial communities. The pyrosequencing data support the hypothesis that the epilimnia of LL and WG Lakes contain distinct bacterial communities ($p<0.01$).

Table 2-1. Lake attributes. Nutrient data was collected several times each year for three years (2007-2009). Shown are means ± one standard deviation.

Lake	Area (acres)	PO_4^- (μg/L)	TDP (μ/L)	TP (μg/L)	NO_3^- (mg/L)	NH_4^+ (μg/L)
Little Long Lake (LL)	170	0.68±0.54	5.8±2.3	9.9±2.1	1.1±0.60	100±63
Wintergreen Lake (WG)	39	14±13	36±15	80±41	0.058±0.14	47±55

Microbial specialization on P compounds

The high mean and narrow variance of the Levins index (mean 11.75 ± 2.59, 1 sd) confirms that many isolates had a broad P-niche breadth, able to use many of the P sources for growth, while others could only use a few of the P sources. Similarly, while most isolates demonstrated similar growth rates across their usable P sources, some isolates grew quickly on one or a few P sources but far more slowly on others. (P-use 'generalists' and 'specialists', respectively; see Figure 2-1).

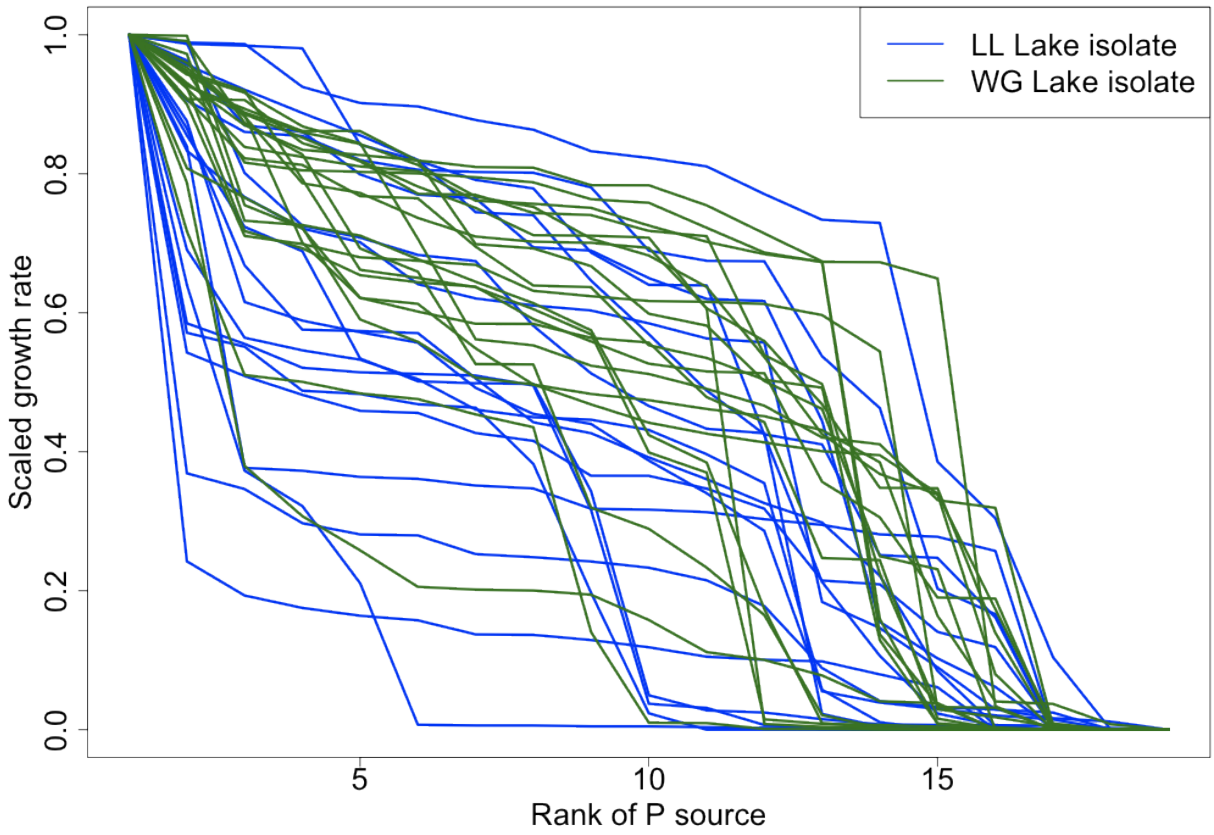

Figure 2-1. Ranked growth rates for each isolate. Each line represents one isolate's scaled growth rates in decreasing order of magnitude. Blue and green lines represent isolates from LL Lake and WG Lake, respectively. Isolates with shallower initial slopes grow relatively well on many P sources ('generalists'); while those with steep initial slopes grow quickly on one or a few P sources and slowly on others ('specialists').

In accordance with our prediction, we found evidence for a tradeoff between max GR and niche breadth. On average, isolates with a broader niche breadth had lower max GRs than those with a narrower niche breadth. [$\log_{10}$(max GR) ~ lake origin*levins index; interaction - $F_{1,35} = 5.48$, $P = 0.025$; levins index - $F_{1,35} = 5.29$, $P = 0.028$; lake origin - $F_{1,35} = 3.26$, $P = 0.079$]. However, the significant interaction between isolate lake origin and niche breadth indicates that this tradeoff is present in only one of the two

lakes—WG Lake, as shown in Figure 2-1. Isolate P-niche breadth accounted for 30-38% of the variation in WG Lake isolates' maximum GRs (regression coefficients with and without phylogenetic correction, respectively), with a broader niche breadth predictive of a lower than average max GR. However, LL Lake isolates' niche breadths were independent of their max GRs, therefore lacking the tradeoff found for WG Lake isolates. This is also supported by analyses that include correction for shared phylogenetic history (WG Lake tradeoff p<<0.01, R = 0.30; LL Lake tradeoff not significant; Phylogeny shown in Figure A-1 of Appendix A).

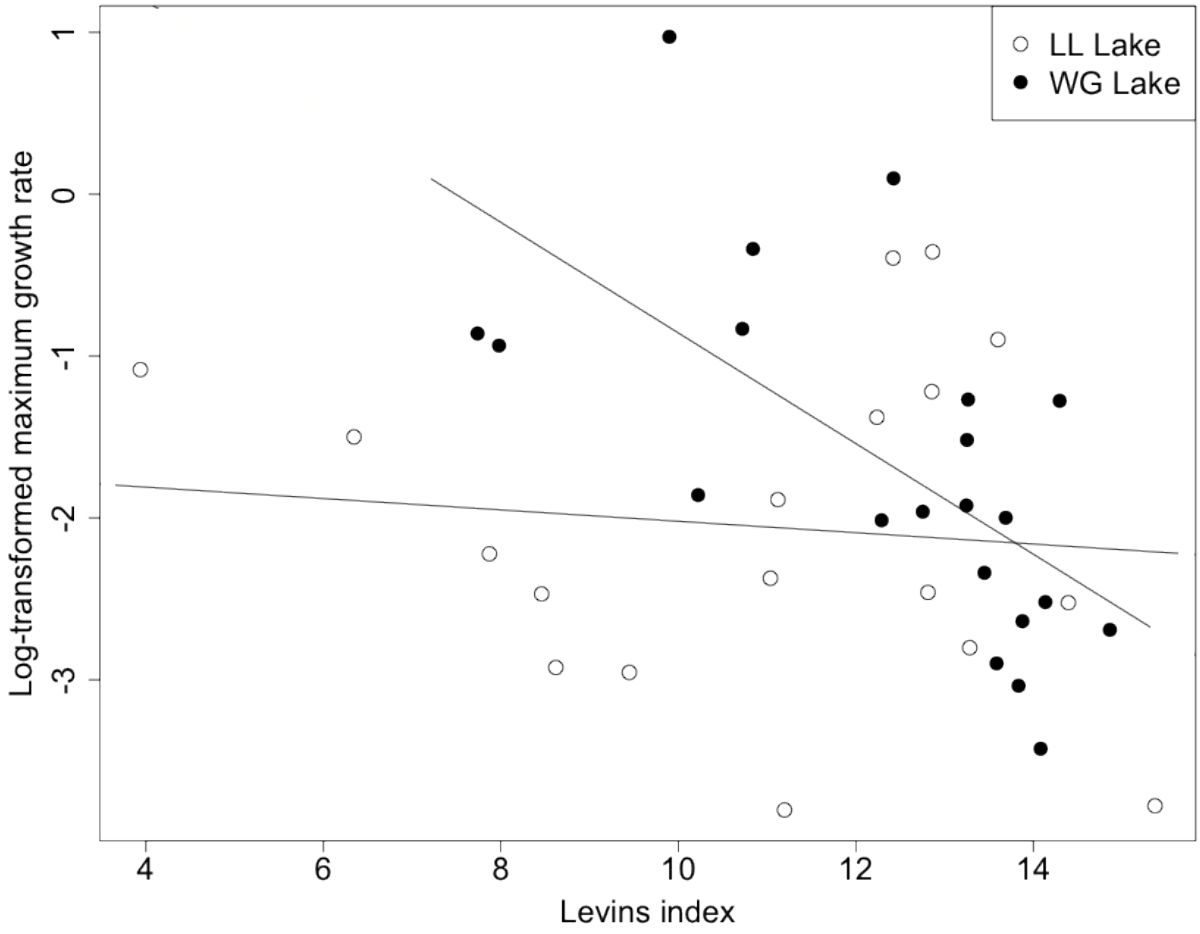

Figure 2-2. Relationship between P-niche breadth and bacterial growth rate. Log-transformed maximum growth rates are plotted against the Levins index for each isolate. Open circles represent isolates from LL Lake. Closed circles represent isolates from WG Lake. Projected slopes in this figure include phylogenetic correction. R2 of regression with phylogenetic correction is 0.30. R2 of regression without phylogenetic correction is 0.38.

We found that the bacterial isolates tended to grow faster on certain P forms. Isolates grew faster on resources that can be accessed without the need for specialized enzymes, such as P_i or nucleotides when compared with resources that do, such as the phosphonate AEP (See Table 2-2 and Figure 2-3; paired t-tests with standardized growth rates, 38 df, $p<<0.05$ for both comparisons). Most isolates grew fastest on TPP, followed by GDP (See Table 2-2 for these and all other P compounds abbreviations). While few isolates grew fastest on P_i, on average, there was no difference in growth rates between P_i and TPP (Paired t-test, 38 df, $p>0.05$). Isolates from both lakes tended to grow fastest on the same resources and slowest on the same resources (data not shown, but see Figure 2-3), with one notable exception— P_i. Isolates from WG Lake had higher standardized growth rates on P_i than those from LL Lake (Two sample t-test with equal variances, 37 df, $p<<0.05$).

Table 2-2. Phosphorus source abbreviations and properties.

Compound Name	Abbreviation	MW	mol P: mol compound	P bond types	Environmental sources
(2-aminoethyl) phosphonic acid	AEP	125.06	1	C-P	bacteria
phytate	Phyt	660.04	6	C-O-P monoester	major P storage form in plants
inorganic phosphate	P_i	174.18	1	phosphoric acid ester	apatite, P_{org} hydrolysis
adenosine-3',5'-cyclic monophosphate	cAMP	351.2	1	C-O-P diester	living organisms
adenosine-5'-triphosphate	ATP	551.1	3	C-O-P monoester & 2 P anhydrides	DNA
phenylphosphonic acid	PhenCP	158.09	1	C-P	herbicide
triphosphate	Poly-P	367.86	3	phosphate esters	major P storage form in bacteria
guanosine-5'-diphosphate	GDP	541.21	2	C-O-P monoester & 1 P anhydride	DNA
guanosine-5'-triphosphate	GTP	523.18	3	C-O-P monoester & 2 P anhydrides	DNA
phospho(enol) pyruvate	PEP	208.04	1	C-O-P monoester	living organisms; intermediate in glycolysis
alpha-D-glucose 1-phosphate	G1P	304.1	1	C-O-P monoester	animals; glycogenesis intermediate
D-glucose 6-phosphate	G6P	282.12	1	C-O-P monoester	living organisms; involved in many metabolic pathways
methylphosphonic acid	MeCP	96.02	1	C-P	biological precurser & degradation product
beta-glycerophosphate	BGP	216.04	1	C-O-P monoester	vertebrates
deoxyribonucleic acid	DNA	608.93	4	C-O-P diester	DNA
L-alpha-phosphatidylethanolamine	Peth	744.05	1	C-O-P diester	bacterial membrane phospholipids
L-alpha-phosphatidylcholine	Pchol	760.09	1	C-O-P diester	animal membrane phospholipids
thiamine pyrophosphate	TPP	460.77	2	C-O-P monoester & 1 P anhydride	synthesized by bacteria, fungi, and plants; required for all organisms

Figure 2-3. Heatmap visually displaying isolate maximum growth rates, scaled from 0-1. Darker colors

Figure 2-3 (cont'd)

represent higher values. The dendrogram clusters isolates and P sources according to these growth rates using the Euclidean method to calculate distances. Isolates are named according to the genus of each as identified using the Classifier tool provided by the Ribosomal Database Project (RDP, Wang *et al.* 2007), numbered when multiple from one genus occur, and are labeled with an "L" or "W" indicating from which lake they were isolated (LL Lake or WG Lake, respectively). P compound abbreviations are found in Table 2.

The isolate trait data confirms some of our expectations for how similar an isolate's GRs should be on various P sources, based on similarity of compound structure. For example, as shown in Figure 2-4, GDP and GTP consistently yield the most similar GRs for a give isolate and in fact, are indistinguishable from each other in this analysis. The two phospholipids and two of the phosphonates also cluster together closely. However, we were surprised to find that PolyP clusters more closely with P_{org} compounds than with P_i, the only other inorganic P source in this study. Some clustering metrics found DNA to be in an indistinguishable cluster from MeCP and PhenCP. This is likely due to the large variance in DNA GR values, and generally poor growth of most bacteria on these P sources.

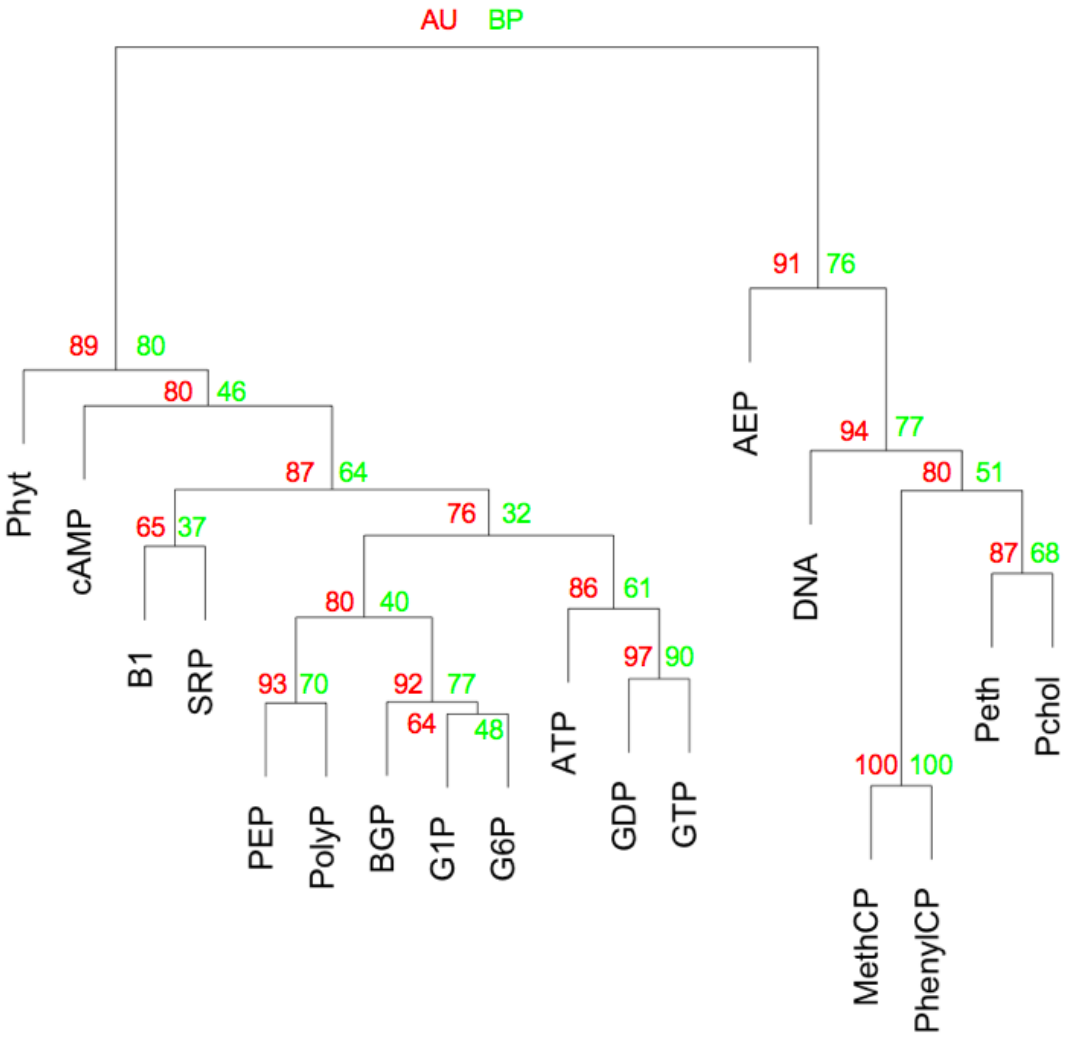

Figure 2-4. P sources clustered according to similarity of P use traits across isolates. The Manhattan method was used to calculate the distance matrix, and the Group Average method with 10,000 bootstrapped permutations was used to cluster the isolates. Green and red numbers indicate the bootstrap probability and the 'Approximately Unbiased' (AU) probability that the cluster exists. Red boxes surround groups for which the AU p-value is less than 0.05, indicating that we should reject the null hypothesis that the clusters do not exist. Thus the compounds within the red boxes are not distinguishable from one another using these metrics.

Importantly, our phylogenetic analyses confirmed that we can consider the isolates to be independent replicates when comparing most P-use traits. While phylogenetic history minimally influences P-niche breadth and growth on AEP and Phyt, bacterial growth rates on cAMP, DNA, and Pchol suggest these P-use traits have

evolved as expected for vertical gene transfer, given the tree topology and model of evolution. We did not find evidence that phylogenetic history affected any other P-use traits (see Table A-1 in Appendix A). Further, the high estimated delta parameter ML values for all traits is indicative of a species-specific mode of trait evolution, while the zero or near-zero estimated maximum value of the scaling parameter Kappa for all traits is consistent with a punctuational, rather than a gradual mode of evolution. These results indicate that phylogenetic history constrains few of the tested P-use traits.

Discussion

The phosphorus resource pool is diverse and plays an important role in determining ecosystem productivity. Recent studies have demonstrated the potential for niche variation according to nutrient use abilities (Martiny *et al.* 2009). Here, we have used physiological assays with environmental isolates to demonstrate that aquatic bacteria vary in their P-use niche breadth, and that this variation can be the basis for a specialization-performance tradeoff.

While most bacteria had a broad P-niche breadth, some tended towards specialization on one or a few P forms. Isolates even specialized on compounds known to be degraded most efficiently by substrate-specific enzymes, such as AEP and Phyt. Though typically considered to be less-accessible forms of P, these compounds have been shown to be readily metabolized by some bacterial groups and have been suggested to play important roles in P metabolism in both aquatic and terrestrial ecosystems (Rodríguez & Fraga 1999, Orchard *et al*. 2009). As the primary P storage form in plants and a significant pool of P in manure, bacterial Phyt degradation has

been suggested to be an important mechanism by which P_{org} from agricultural lands is made labile across a landscape, traveling from farms to nearby water bodies where excess P causes unwanted algal blooms (Hill *et al.* 2007). The widespread ability of our isolates to access P from and even specialize on Phyt suggests that P liberation from this compound may not only be important in P transport across terrestrial environments, but likely continues in aquatic systems.

Our results support the system-specific nature of performance tradeoffs. WG Lake isolates with a broader niche grew more slowly than those with a narrower niche breadth, yet there was no such tradeoff among LL Lake isolates. There are many possible causes of this disparity. More productive environments may confer the greatest benefit to niche-specialization by both increasing specialization opportunity on the most abundant resources and increasing availability of rare resources (Futuyma & Moreno 1988, Chow *et al*. 2004). Increased availability of a variety of resources may effectively make WG Lake a more homogeneous environment with respect to the P resource needs of any given bacterial strain. If this is true, and WG Lake bacteria are frequently limited or co-limited by resources other than P, then P-use generalists in WG Lake may be unnecessarily expending more energy to meet their P resource needs than their specialist counterparts. Alternatively, differences in nutrient acquisition among LL isolates may be minimized in productive environments like those of the experimental environment, and any advantage of niche-specialization may only be apparent under conditions of nutrient scarcity, more closely resembling the environment from which the strains were isolated (Jessup & Bohannan 2008, Buckling *et al.* 2007). On the other hand, LL Lake isolates may trade off traits in favor of niche-generalization that were not

measured in this study, such as expending energy to maintain a larger genome size. Finally, generalists do not necessarily experience a cost for maintaining a broad niche-breadth, and this may be the case for the LL Lake isolates (Buckling *et al.* 2007).

The isolates' ecological history primarily constrained the P-niche breadth/ GR tradeoff, while their phylogenetic history likely constrains only a few P-use traits. Accounting for phylogenetic history only minimally reduced the effect size of the ecological tradeoff in WG Lake. This indicates that ecological or environmental factors such as differences in lake bacterial community composition, nutrient concentrations, or abundances of specific P forms may primarily influence the strength of this ecological tradeoff. Consistent with other studies, we found substantial strain-specific variation in P-use for nearly all traits (Huang *et al.* 2005, Martiny *et al.* 2006). That most trait data did not correspond well with the phylogeny may be a reflection of complex and variable genetic regulation, repeated independent evolution of many traits, or possibly the lateral acquisition of P-use genes (Martiny *et al*. 2006; Martiny *et al*. 2009). Yet that some P-use traits were at least minimally influenced by phylogenetic history suggests that they may be phylogenetically constrained. These traits included those enhanced by one or more specialized enzymes, such as phosphonate, phytate, cAMP and DNA metabolism. If these traits are phylogenetically constrained, community composition may ultimately limit P turnover of these compounds in an ecosystem.

Our physiological data support the importance of diverse P sources for meeting bacterial community P demand. Isolates in our study differentially grew on P compounds with similar compound structures, such as the nucleotides GTP and ATP. This measurable physiological response to relatively small differences in P resource

form may be an effect of a number of factors. Even small structural differences between the compounds may affect the ability for enzymes to cleave phosphate from a molecule; bacteria may be able to access both nitrogen and phosphorus from some compounds; or bacteria may save different amounts of energy by taking up whole or partial P-containing structural components rather than synthesizing organic compounds from their inorganic building blocks. Though the P source on which the isolates most commonly grew fastest was TPP, a vitamin with an easy-to-degrade pyrophosphate group, many also grew fastest given GTP or GDP as their sole P source. Several studies have indicated that nucleotides may be among the most readily available P_{org} sources, particularly in oligotrophic environments, taken up and regenerated up to five times more quickly than the bulk dissolved P_{org} pool (Cotner & Wetzel 1991; Siuda & Chróst 2001, Karl and Barkman 2005, Lennon 2007 and references within). If bacteria in natural communities gain an advantage for fast growth on particular P sources, then the dominant P form in aquatic environments may significantly affect microbial community structure and species dominance.

This study provides evidence that nutrient-based resource specialization can significantly influence important performance traits of an organism, such as its growth rate, though these effects may be system-specific. Additionally, organic nutrient forms may be more important in structuring community and ecosystem dynamics than previously thought.

APPENDICES

APPENDIX A

Supplementary Figures

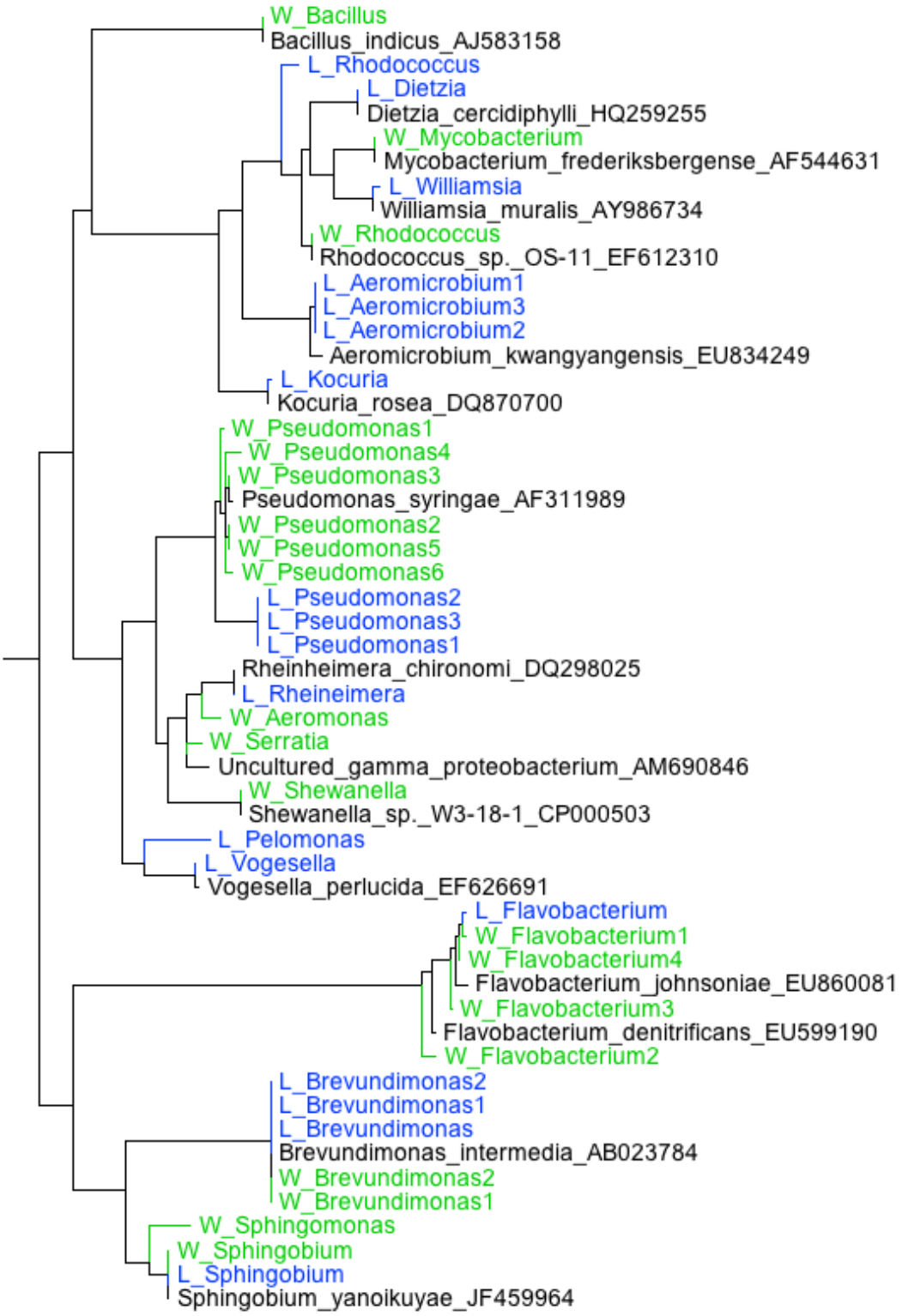

Figure A-1. Phylogeny of bacterial isolates used in this study, with reference sequences. As in Figure 2-3, isolates from the present study are named according to the genus of each, as determined from RDP

Figure A-1 (cont'd)

classification, numbered when multiple from one genus occur, and are labeled with an "L" or "W" indicating from which lake they were isolated (LL Lake or WG Lake, respectively). They are also color-coded by lake—LL Lake isolates are blue and WG Lake isolates are green. Reference sequences (in black) are named according to their RDP or genbank classification, when an RDP classification was unavailable. All reference sequences are also labeled with their Genbank identifier.

Table A-1. Influence of phylogenetic history on phosphorus-use traits. ML = Maximum likelihood. For analysis details and compound abbreviations, see Methods section and Table 2-2 from text, respectively. Within the software program BayesTraits, one can test whether a phylogeny correctly predicts species trait covariances by incorporating the parameter Lambda (l) in analyses (Pagel 1997, Pagel 1999). Significant P-values for l= 0 vs. ML comparisons indicate that phylogenetic history at least minimally influences the trait. Significant P-values for l= 1 vs. ML comparisons indicate that the trait is not perfectly correlated with the phylogeny. In this study, we considered traits to be phylogenetically conserved if >95% of generated phylogenetic trees statistically support phylogenetic conservatism (a= 0.05). For example, a trait that perfectly correlates with the phylogeny would yield a '100' in the first column and a '0' in the second; while one that is minimally influenced by phylogenetic history would yield a 95-100 in the first column and a value 5 or greater in the second. Values supporting phylogenetic conservatism are starred.

Phosphorus compound	$\lambda = 0$ vs. ML value % of trees with P-values <0.05	$\lambda = 1$ vs. ML value % of trees with P-values <0.05
AEP	27	32
Phyt	100*	0.55*
P_i	0	90
cAMP	100*	2*
ATP	0	100
PhenCP	0	100
PolyP	0.10	99
GDP	0.0	100
GTP	0.0	100
G1P	0.0	72
G6P	0.0	94
MeCP	0.0	98
BGP	24	13
DNA	100*	13
Peth	0.0	100
Pchol	100*	21
TPP	0.70	86
Levins	0.0	100

Figure A-2. Isolate 16S DNA sequences used for phylogenetic analyses. Sequences are shown in FASTA format.

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Figure A-2 (cont'd)

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Figure A-2 (cont'd)

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Figure A-2 (cont'd)

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Figure A-2 (cont'd)

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Figure A-2 (cont'd)

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Figure A-2 (cont'd)

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Figure A-2 (cont'd)

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Figure A-2 (cont'd)

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Figure A-2 (cont'd)

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Figure A-2 (cont'd)

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Figure A-2 (cont'd)

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Figure A-2 (cont'd)

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APPENDIX B

Supplementary Methods

Final Contents of Phosphorus-Defined Media

Chemical	Final Concentration (μM)
$CaCl_2 \cdot 2H_2O$	250
$MgSO_4 \cdot 7H_2O$	150
$NaHCO_3$	150
NH_4Cl	250
KNO_3	250
$CuSO_4 \cdot 5H_2O$	0.04
$ZnSO4 \cdot 7H_2O$	0.08
$CoCl_2 \cdot 6H_2O$	0.04
$MnCl_2 \cdot 4H_2O$	0.91
$NH_4Mo_7O_{24} \cdot 4H_2O$	0.03
$FeCl_3 \cdot 6H_2O$	12
H_3BO_3	2.1
$Na_2EDTA \cdot H_2O$	4.36mg/L
H_2O_3Se	0.6
HEPES buffer	2.38g/L

Defined Media Recipe

Chemical	Final Concentration
1000X Major Elements Working Stock	1X
1000X Trace Elements Working Stock	1X
1000X Vitamin Working Stock	1X
100X Carbon Source Stock*	1X
Phosphorus source (see Table 2-1)*	Variable. See text for details.
HEPES buffer	2.38g/L
Cyclohexamide*	50 mg/L

*Filter-sterilized.

WORKING STOCK SOLUTIONS

1000X Major Elements Working Stock

Chemical	Final Concentration (mM)
$CaCl_2 \cdot 2H_2O$	250
$MgSO_4 \cdot 7H_2O$	150
$NaHCO_3$	150
NH_4Cl	250
KNO_3	250
H_2O_3Se	0.6
H_3BO_3	2.1

1000X Trace Elements Working Stock

Chemical	Final Concentration (mM)
$CuSO_4 \cdot 5H_2O$	0.04
$FeCl_3 \cdot 6H_2O$	12
$CoCl_2 \cdot 6H_2O$	0.04
$MnCl_2 \cdot 4H_2O$	0.91
$NH_4Mo_7O_{24} \cdot 4H_2O$	0.03
$ZnSO4 \cdot 7H_2O$	0.08
$Na_2EDTA \cdot H_2O$	4.36g/L

Vitamin Working Stock

Chemical	Final Concentration (mg/L)
Biotin	1.0
Thiamine HCl	200

Carbon Source Working Stock

Chemical	Final Concentration (g/L)
Glycine	31.25
Acetate	31.25
Dextrose	93.75
NaSuccinate • $6H_2O$	93.75

PRIMARY STOCK SOLUTIONS

Chemical	Stock Concentration (mM)
$CaCl_2 • 2H_2O$	250
$MgSO_4 • 7H_2O$	150
$NaHCO_3$	150
NH_4Cl	500
KNO_3	500
H_2O_3Se	0.6
$CuSO_4 • 5H_2O$	40
$ZnSO4 • 7H_2O$	80
$CoCl_2 • 6H_2O$	40
$MnCl_2 • 4H_2O$	910
$NH_4Mo_7O_{24} • 4H_2O$	30
H_3BO_3	2.1
Biotin	0.10 g/L
Cyclohexamide	25 g/L

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CHAPTER 3

FUTURE DIRECTIONS

P form may have large impacts on community and ecosystem properties, but these are neither quantified nor well understood. The present study was an important first step in understanding how P form can impact the life history of bacteria, whose uptake and transformation of dissolved P is crucial for nutrient cycling. Yet completion of a few follow-up research studies could greatly increase our understanding of the impacts of P form in natural systems. Do bacteria demonstrate niche partitioning according to P form or abilities? Can P form significantly influence community composition and ecosystem functions? Experimental results from studies addressing these questions would contribute greatly to increase our understanding of the maintenance of species diversity, community dynamics, and ecosystem functioning.

Since bacteria vary greatly in their ability to use diverse P forms and demonstrate the ability to specialize on P forms, it seems reasonable that they may also partition P resources. Resource partitioning through niche differentiation can facilitate species coexistence, thereby increasing species diversity (Chesson 2009). One can experimentally test for the ability to partition resources by conducting a series of competition experiments in chemostats with different P environments. For these experiments, two isolates should be chosen such that each performs best on a different P source and has a clear disadvantage on the P source the other performs best on. For example, W_Pseudomonas1 and L_Psuedomonas2 would be good candidates, since W_Pseudomonas1 grows very well on AEP, but poorly on Phyt, while L_Psuedomonas2 grows well on Phyt, but poorly on AEP. Positive evidence for niche

partitioning would be found if isolates competitively exclude each other when grown in the most advantageous P environment of each, but coexist when grown with both P sources available.

P form influences bacterial performance traits and ecology, but can it also have broader-scale impacts? The often substantial effects of inorganic phosphate additions on community dynamics and ecosystem functions are well documented (Carpenter *et al*. 1998, Smith 2003). However, theoretically all members of plant, algal, and bacterial communities can access P_i, while access to P resources from P_{org} is likely predominantly mediated by bacteria. In P-limited environments, this possible shift to a bacterial-controlled P limitation may yield different effects on communities and ecosystem functions. Yet few studies have included diverse phosphorus sources when comparing community or ecosystem impacts, limiting our understanding of the importance of P form at these scales.

A mesoscale experiment investigating the community and ecosystem impacts of diverse P forms would be a valuable contribution. For example, cattle tanks could easily be used as replicated aquatic mesocosms. These mesocosms could be 'seeded' with microbes and macroinvertebrates from local lakes and allowed to reach a stable state over time. Several different P forms, such as ATP, Phyt, AEP, and P_i could be added. A mixed P treatment with equal concentrations of each added P form could provide valuable insight into broad-scale effects of P resource diversity. Ecosystem-level variables could then be quantified, such as ecosystem respiration and primary productivity, nutrient concentrations and stoichiometries, and total biomass across trophic levels (here, microbes and macroinvertebrates). Measured community-level

responses could include bacterial respiration and productivity (and thus growth efficiencies), and bacterial and zooplankton community compositions and diversity.

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