Diversity and abundance of inositol phosphate utilizing bacteria in marine and freshwater sediments and forest soils of Cape Cod

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Abstract

Inositol phosphate (IP) is a dominant fraction of organic phosphorus in soils. Microbial utilization of IP is poorly understood. The goal of this study was to evaluate the diversity and abundance of IP utilizing bacterial in forest soils, and salt and freshwater marshes. We cultured IP-utilizing bacteria from environmental samples using a variety of culturing conditions on agar plates with IP as the sole phosphorus source and cloning techniques to determine phylogenetic diversity of culturable bacteria. The freshwater marsh contained the highest abundance and diversity of bacteria that can use IP as a sole P source compared to forest soils and a marine marsh. The forest soil community represented a distinct assemblage compared to the marine and freshwater marshes, whereas the marine community was a subset of the taxa found in the freshwater marsh.

Introduction

Inositol Phosphate Utilizers

Inositol phosphates (IP) are ubiquitous, organic compounds in the environment (Turner et al., 2002). In soil environments, organic phosphorus (P) is often a large fraction of the total P reserve, and IP can comprise up to 90% of the organic P pool (Turner et al., 2002). Organic detritus from plants is the dominant source of IP inputs to soil environments. IP is also present in freshwater and marine sediments, where it is produced *in situ* or delivered as sediments in runoff from terrestrial ecosystems (Suzumura and Kamatani, 1995b). The accumulation of large pools of IP in soils suggests limited bioavailability and that the mineralization of IP is a critical "bottleneck" in the organic P cycle. However, IP is apparently quickly mineralized in coastal and marine ecosystems (Suzumura and Kamatani, 1995a), where it may contribute to eutrophication.

Because P availability may limit productivity in both terrestrial and aquatic ecosystems, the ability to utilize IP may confer competitive advantages. Plants, fungi and microorganisms may contain the enzyme phytase that liberates ester-linked phosphates from the six carbon ring inositol. Even though phytases are found in plants, utilization of IP by plants is greatly increased in the presence of soil bacteria (Richardson et al., 2001), suggesting that the ability to use IP is not a universal trait and that microorganisms may be responsible for the bulk IP mineralization in all ecosystems. Moreover, there is evidence that the ability to mineralize IP depends upon environmental conditions as well as the microbial community. In marine sediments incubated both aerobically and anaerobically, the dephosphorylation of IP and concomitant release of inorganic P was twice as fast under anaerobic conditions (Suzumura and Kamatani, 1995a).

Understanding the diversity of microorganisms that can use IP and the conditions under which this is favorable is an important step for understanding the dynamics of the global phosphorus cycle. The first objective of this study was to compare the abundance and richness of bacteria that can utilize IP as a sole P source in upland forest soils, and freshwater and marine sediments. Our approach was to collect environmental samples from different habitats and culture microorganisms on appropriate media containing *myo*-inositol hexaphosphoric acid as the sole P source. To understand how variation in the abiotic environment might influence the abundances of IP-utilizing microbes, samples from each habitat were cultured under different conditions. Phylotype richness of these isolates was assayed by generating clone libraries from PCR-amplified 16S rRNA genes and using aligning and treeing techniques to describe the organisms represented by the amplicons. Abundance was quantified in most probable number dilution series.

Phytase

Phytases (*myo*- inositol hexokisphosphate phosphohydrolase) are a class of enzymes thought to occur widely in nature, although very little is known about the occurrence of these enzymes in natural environments. These enzymes potentially play an important role in phosphorus cycling due to their role in inositol phosphate degradation. Phytases differ from phosphotases due to an ability to hydrolyze the very stable monophosphoester bond of inositol phosphate releasing inorganic phosphate. Inhibition of phytase synthesis at high concentrations

of inorganic phosphate suggests a high metabolic cost of production (Shieh and Ware, 1968) and may limit diversity of organisms producing phytase in nature. Representative phytase enzymes from plant, fungal and bacteria groups have been cloned and characterized. Although within each of these groups few proteins have been isolated and characterized, a probable conservative motif has been identified which is shared among all characterized phytases (Ullah et al. 1991). Due to the common occurrence of inositol phosphate in plant derived stock feed and the anti-nutritive effect associated with IP, most work related to microbial phytase production and occurrence in microbial populations focuses on industrial use. Consequently, phytase occurrence in environmental microbial communities has been neglected.

Two broad classes of phytases occur in nature, 3-phytase and 6-phytase; 3-phytase is typically associated with microorganisms while 6-phytase is typically associated with plants. 3-Phytase has been detected and described in isolates of fluorescent *Pseudomonas* spp. and non-fluroescent *Pseudomonas* spp., E. coli, Bacillus sp, Klebsiella spp. Psuedomonas sp., Enterobacter, and Citrobacter spp. (summarized in Kerovuo 2000). The use of a PCR primer set for phytase as a qualitative assessment of IP degrading communities has not been previously described. The second objective of our study was to test degenerate primers of characterized phytase genes for the potential use of detecting IP utilizing bacteria. It is likely that future investigations of phytase in natural populations will use such a cultivation-independent approach to understand the diversity of environmental IP utilizing communities.

Methods

In order to maximize the diversity of organisms recovered from culturing, we tried to "cast a wide net". Thus all minimal media contained glucose as a carbon source. We assumed that the majority of the microbes in surface soils of forests may be cultured aerobically but have different pH ranges, so forest soil samples were incubated in media at pH 5.4 and 7.0. We further assumed that surface sediments from freshwater and marine marshes experience fluctuating oxygen concentrations, so samples from these habitats were incubated both aerobically and anaerobically in media at pH 7.0.

Site Descriptions and Sampling

All samples were collected from the environs of Woods Hole, Massachusetts. The forest site, Beebe Woods, is a second growth forest with an overstory of red oak, white oak and pine, and an understory dominated by huckleberry. The soils in Beebe woods are developed from glacial moraine parent material and tend to have sandy loam texture. The freshwater marsh, School St. Marsh, was dominated by *Spartina patens*, which formed a dense organic mat that essentially floated on the surface of the water. Last, we collected samples from a marine salt marsh, the Little Sippewissett dominated by *Spartina alterniflora*.

All soil and sediments samples were collected using similar methods. At each site three sampling points were located 10 to 15 m apart. Each sampling point consisted of 5 bulked subsamples from a 1 m^2 area. Samples were extracted with a 2 cm diameter corer to 5 cm depth in the mineral soil or sediment layer (i.e. excluding the forest floor and surface organic material). The sampler was wiped clean with 70% ethanol in between samples to minimize cross-contamination. This coring method worked well for all sites except the School St. Marsh. Here we collected samples of the dense organic mat from the top 5 cm of shovel samples.

Media and Cultivation

Media specific for the three environments (forest soil, freshwater sediments and marine sediments) were prepared following standard protocols (Appendices 1-3). For the different media, *myo*-inositol hexaphosphoric acid from corn (Sigma Aldrich, CAS 14306-25-3) was the sole P source.

Two g samples of field moist, homogenized soil or sediments were shaken in 20 mL volumes of appropriate liquid media (without a P source) for 15 minutes, allowed to settle for 15 minutes, and then diluted 10^0 to 10^{-5} in the appropriate liquid media containing IP. For the dilution series, 0.1 ml of each solution was spread onto agar plates containing appropriate media. As a positive control, one plate with inorganic phosphate as the sole P source was inoculated with 0.1 mL of the 10^0 dilution for each combination of site and treatment (18 plates total). We expected diverse colonies and abundant growth on these plates. As a negative control, one P-free plate per treatment and site combination was inoculated (18 plates total). We also incubated P-free agar plates that were not inoculated. Anaerobic plates were incubated in air-tight containers scrubbed of oxygen by BBL GasPack Anaerobic System envelopes (Becton Dickinson). All plates were incubated at 30 °C and monitored for colony formation. Subsamples of the soils/sediments were dried at 110°C in order to express colony forming unit (CFU) counts on a dry weight basis.

Because there may have been small amounts of available phosphate present in the initial inoculum or in the *myo*-inositol hexaphosphoric acid, we measured phosphate concentrations in all dilution series of inocula using the malachite green method (Lanzetta et al., 1979).

Microbial Abundance

We used most probable number (MPN) techniques to estimate abundances of culturable colony forming units from the dilution series of agar plates (Fredrickson and Balkwill, 1998). We selected plates with between 19-370 colonies prior to plate wash and DNA extraction (after 8 days of incubation) and counted the number of total colonies on these plates taking the dilution factor into account. Abundances of colony forming units are reported as a function of oven dry soil or sediment mass (oven dried at ~110 °C for 48 hours).

We used microscopy to estimate total abundances of non-culturable bacteria in each habitat. Samples of the supernatant from the initial soil or sediment extractions were diluted 1:1 with ethanol to preserve them for direct cell counts. We passed the extractions through Whatman #1 filter papers (20-25 micron) to remove sand particles and debris before filtering 6 ml aliquots through 0.2 μm polycarbonate membrane filters for staining and hybridization. Cells on the filters were hybridized with horseradish peroxidase-labeled oligonucleotide rRNA probes specific for Eubacteria (Table 1) using standard protocols developed for catalyzed reporter deposition (CARD-FISH) (Sekar et al., 2003). Direct cell counts were made at 100X magnification using fluorescent microscopy. Approximately 1000 cells were counted in five microscope fields. Results are expressed as a function of oven dry soil or sediment mass.

Richness of IP Utilizing Bacteria

For each of the 18 samples (three habitats, three replicates per habitat, and two culturing conditions), 16S rRNA genes were amplified using bacteria specific primers (8F and 1492R) from washes of plates containing the greatest numbers and morphological diversity of colonies. We used the MoBio Ultraclean Fecal kit for DNA extraction (Solona Beach, CA). In all cases, two or three plates from each of the 18 dilution series, excluding plates with undiluted inocula, were washed with 500-600 µL Bead Solution, the colonies were aggressively detached from the agar with a sterile glass spreader, and cells were transferred to a screw-cap centrifuge tube by pipetting. We did not use samples from the first plates in the dilution series because small amounts of phosphorus (inorganic or organic) may have been added to these plates in the initial inoculum. 16S rRNA genes were PCR amplified from extracted DNA in 25 µl reactions of 2.5 µl PCR buffer solution, 0.4mM dNTP, 2mM $MgCl_2$, 0.4µM 8F and 0.4 µM 1492R, and 0.5 µl *Taq* DNA polymerase. We amplified 16S rDNA fragments using the following parameters: initial denaturing at 95 °C for 5m, and 25 cycles of 30s denaturing at 95 °C, 30s of annealing at 55 °C, and 1m of extension at 72 °C. PCR products were pooled according to environment and incubation condition (e.g. all three replicates of forest soil, pH 5). Pooled amplified fragments from soil environments were ligated into TOPO plasmid vector and transformed into competent E. coli cells (Invitrogen Co., Carlsbad, Calif.). Pooled PCR products from the other two habitats were ligated two days later and thus incubated with an additional Taq polymerase and dNTP at 72 °C for 5m to attach A's onto fragment ends, then ligated into TOP-TA plasmid vector, transformed into competent E. coli cells (Invitrogen Co., Carlsbad, Calif.) and screened using blue/white cell distinction. This method produced six clone libraries each represented by 48 clones. Clones plasmids were prepped and sequenced using the automated sequencing facility at the Marine Biological Laboratory, Woods Hole, MA.

Resulting sequences were checked for quality and placed within a phylogenetic context. Sequences were aligned in ARB and compared to a large database of 16S sequences. Because we amplified a small region of the 16S rRNA gene (~420 BP) we cannot use these data to construct a robust phylogeny. Instead, we used the data to determine the most closely related sequences to our clones and to construct a tentative tree of the phylogenetic relationships among clones.

Primer Design and Amplification of Phytase Gene DNA

Degenerate phytase primers were designed using amino acid sequences from six bacteria species with cloned and characterized phytase genes. We used web-based searchable databases, BLAST (NCBI) and InterPro (EMBL), to identify phytase gene sequences of cloned and characterized genes. We used the amino acid sequences of the phytase gene from the following target species: *Bacillus subtilis*, *B. licheniforms*, *B. amyloliquefaciens*, *B.* strain D01, *Pseudomonas syringae*, and *Shewanella oneidensis*. These species were used based on shown expression of cloned phytase gene. We aligned amino acid sequences and identified conserved reagions using Blockmaker and generated consensus degenerate primers using CodeHop (Rose et al., 2003). Two forward primers and two reverse primers were identified and ordered (Table 2).

16S PCR amplification was used to screen DNA extractions for primer use. We extracted environmental DNA from the pooled inocula used for plating for each environment type using the bead beating method described above (Mo Bio UltraClean Soil DNA Isolation Kit

and protocol; MO BIO Laboratories, Inc.,). In addition, we tagged twenty isolates among the plates used for cultivating IP utilizers based on unique morphology for DNA extraction. Cells of isolates were lysed by boiling cells for 5 minutes in DMSO and 0.05% Triton X. Potential actinobacteria were put through a series of freeze-thaw cycles. Minimal success in achieving PCR amplification using the parameters described above was achieved. Using the primer set, Phy 2B3 and Phy 3D5, attempts at amplifying phytase genes from both environmental DNA and isolate DNA failed. We used standard 25 μl reactions with 0.5μM primers and put reactions through 30 cycles of denaturing at 94° C for 45s, annealing across a gradient from 50 to 62 °C for 45s and extending at 72 °C for 1m. No amplification of desired band size occurred. Due to lack of time PCR conditions could not be optimized and requested positive control could not be received in time. We do not discuss these results further.

Soil and Sediment Chemical Properties

pH was measured in water on field-moist soils and sediment with a 1:2.5 soil:solution ratio. Subsamples were oven dried at 34 °C, passed through a 2 mm sieve and will be analyzed for inositol phosphates (Dr. Ben Turner, University of Florida) and total carbon and nitrogen (University of Minnesota).

Statistical Analyses

We used analysis of variance (ANOVA) to determine whether there were differences in the abundance of colony forming units (CFU) among habitat and culturing conditions. For this test, we used habitat x culture conditions as a main effect with six categories (e.g. pH 5.4 agar in forest soils, pH 7 agar in forest soils, aerobic freshwater sediments, anaerobic sediments, etc.). Culture conditions were not nested within habitat because all culturing conditions were not attempted for each environment. *Post hoc* Tukey's mean separations tests were used to assess pairwise differences in mean CFU between combinations of habitat/culture conditions. Residual plots verified that data transformations were not necessary.

Ecological communities may differ in species richness, i.e. the number of taxa present, in several ways. Two communities may: i) have a large number of taxa in common, ii) have few taxa in common, or iii) the taxa in one community may be a subset of the taxa present in the other community. Comparisons of species richness among microbial communities are complicated by inconsistent definitions of what constitutes a species. Recent statistical techniques have been developed that compare the similarity of clone libraries of 16S rRNA gene sequences from microbial communities (Singleton et al., 2001). This method calculates a test statistic referred to as "coverage" that describes the sequence similarity between clone libraries over a range of definitions of similarity, and tests for significance against a distribution of the test statistic obtained by randomly reshuffling the observed data for a large number of iterations. We evaluated the pairwise differences in phylotype richness among the three habitats using this method implemented in the program LIBSHUFF (Singleton et al., 2001). Within the freshwater and marine sediment clone libraries, we also evaluated whether the communities of isolates differed between incubation conditions (aerobic versus anaerobic).

Results

Soil and Sediment Characteristics

The forest soils were significantly more acidic (mean pH = 4.4 ± 0.06 standard error) than the fresh water and marine sediments (6.49 ± 0.23, 6.71 ± 0.02, respectively), which did not differ statistically from one another as a determined by ANOVA and Tukey's means separation tests ($F_{2,6} = 88.14$, $p<0.0001$).

Bacterial Abundances

We observed abundant growth of morphologically diverse colonies on the inorganic phosphate plates from all habitats and culture conditions (positive control). We also observed numerous colonies on the P-free plates (negative controls) under all culture conditions, although these colonies were consistently smaller than colonies on IP or P plates and morphologically similar to one another. We speculate that these may have been growing on the sparing amounts of P present in the initial inocula. Two lines of reasoning support this conclusion: first, there was no colony formation in any of the uninoculated controls, and second, we observed very few colonies in the lowest dilution IP plates.

Abundances of culturable colony forming units that used IP as a sole P source varied from 1.2×10^5 to 3.5×10^6 per gram dry weight. IP-utilizing bacteria were most abundant in the freshwater sediments (Figure 1), and nearly an order of magnitude less abundant in forest soils and marine sediments. Within habitats, there were no statistically significant differences in CFU between cultivation conditions, although the plates incubated anaerobically consistently yielded higher counts in both the marine and freshwater sediments.

The patterns of bacterial abundance identified by Eubacterial CARD FISH probes and counted directly from environmental samples mirrored those from culturing conditions specific for IP utilizers, but were two orders of magnitude higher. On average, there were 7.4×10^8 bacterial cells per gram dry weight in the freshwater marsh samples, 9.5×10^7 cells in forest soils, and 9.0×10^7 cells in the saltwater marsh sediments.

IP Utilizer Richness

Two hundred and five total clones were analyzed for this study. Freshwater and saltwater libraries were each represented by 85 and 89 clones respectively, while only 31 clones represented the forest soil environment. This disparity between the number of soil sequences and those of the other two sequences is due to poor cloning efficiency. The small representation of the bacteria community within the forest soil environment limits assessment of diversity; however, the high proportion of these clones (63% of the forest soil) located together within the Burkholderiaceae suggests that the cultured forest soil IP utilizer diversity is low or poorly characterized using these specific molecular techniques. Of the successfully sequenced clones, the vast majority fell within the proteobacteria division, specifically 46% of clones represent gamma proteobacteria, and 16% in the beta proteobacteria. 32% of the clones are in the arcobacter division all of which are freshwater and saltwater clones. Thus 98% of the clones are represented by three major bacteria lineages (Figure 2). These results suggest that overall the diversity of culturable IP utilizing bacteria may be relatively limited.

Community Composition

The community of phylotypes present in the pooled clone libraries from the soil samples was statistically different from both communities from seawater and freshwater sediments (Table 3). Similarly, both the marine and freshwater communities were also different from the soil librairies, indicating that the soil sequences formed a distinct community with little overlap to either marsh community. In contrast, the clones from the marine sediments were not statistically different from the freshwater sediment clones ($p=0.495$), but the freshwater community was significantly different from the marine community ($p=0.001$). This indicates that the marine taxa were largely a subset of the clones found in the freshwater sediments, which contained additional taxa not present in the marine ecosystem. Within both the marine and freshwater marsh clone libraries, the taxa isolated aerobically were significantly different from those isolated under anaerobic conditions (Table 4).

Discussion

The prevailing view of inositol phosphates is that they are produced largely in terrestrial environments where their bio-availability is limited by abiotic and biotic mechanisms, then transported to marine and coastal ecosystems via erosion and riverflow, where rapid mineralization occurs (Smil, 2000; Turner et al., 2002). We originally conceptualized the three habitats we sampled as forming opposing gradients of IP production (from forest soils to marine sediments) and utilization (from marine sediments to forest soils). In this scheme, we expected that the freshwater marsh may form an intermediate microbial community between the forest soils and marine sediments with respect to composition and abundance of IP utilizers, because the marsh receives litter inputs from the surrounding forest but has anoxic surface waters. Surprisingly, the freshwater marsh contained higher densities of culturable IP-utilizing bacteria and the largest richness of isolates compared to the other two environments.

What is different about the freshwater marsh? While similar to the marine sediments in terms of pH, the freshwater marsh samples were very organic, and had ratios of fresh weight to oven dried weights that ranged from 6.4 to 8.4. The forest soils and marine sediments consisted largely of mineral particles such as sand (with wet to dry weight ratios from 1.24 to 1.67), and normalizing bacterial cell and MPN counts to grams dry weight of sample increased the apparent abundances in the freshwater samples. However, the fact that the rank order of bacterial abundance among communities correlates with diversity suggests that the striking differences between the freshwater marsh on the one hand, and the forest soil and salt water sediments on the other hand is not an artifact. Importantly, the taxa present in the marine sediments were a subset of the freshwater taxa. Thus, freshwater, organic sediments like those at School St. Marsh may be hotspots of both abundance and diversity of bacterial IP utilizers. It is also possible that environmental conditions such as nutrient availability differ among the three habitats and that the abundances and diversity of IP-utilizing bacteria reflect these differences. Plant production in a nearby saltwater marsh dominated by *S. alterniflora* is limited by N availability (Teal and Howes, 1996), which may further limit the importance of IP utilization as an ecologically favorable trait in this environment.

Forest soils differed little from marine marsh sediments in terms of the abundances of IP-utilizing bacteria, although there was almost no overlap in community composition. Even

considering the low number of clones sequenced, forest soils showed very little diversity in culturable IP-utilizing bacteria relative to that found in either aquatic environment. This low diversity also corresponded to a relatively low diversity of colony morphology on plates with innoculum from soil. Many colonies appeared to be Actinobacteria which was not represented in the clones sequenced. The dominance of Burkholderia in the clones sequenced and the dominance of potential Actinobacteria colonies on inoculated IP plates suggest a low diversity of organisms in soil environments that can utilize IP and bias in the DNA amplified by PCR (Wintzingerode et al., 1997). Making an assumption that the low diversity of culturable IP utilizers reflects relative abundance and uncultured diversity of IP utilizers, these results help explain the large accumulation of IP in forest soils. Future studies should include *in situ* hybridization techniques to determine relative abundance of these cultured groups and developing a PCR phytase gene system to determine potential diversity of IP utilizers unidentified by the culturing techniques used in this study.

For both the marine and freshwater sediments, the communities of bacteria isolated using aerobic culturing techniques were distinct from those on plates incubated anaerobically, even though colony abundances did not differ statistically between culturing conditions. Little overlap between the communities in oxic versus anoxic conditions implies that we isolated both obligate aerobes and obligate anaerobes, respectively (and not facultative anaerobes). The coexistence of both obligate aerobes and anaerobes in samples from both marine and freshwater sediments implies microheterogeneity in oxygen conditions. Another surprising result was the similar abundances of bacteria isolated aerobically versus anaerobically. A previous study found faster mineralization of IP in anoxic versus oxic conditions in marine systems (Suzumura and Akiyoshi, 1995a), and thus we expected greater abundances of bacteria under anaerobic culture conditions. The biotic potential of IP mineralization at both the freshwater and saltwater marshes in our study is not dependent on redox conditions. Concurrent study of *in-situ* and potential IP mineralization rates are needed to understand whether IP mineralization is determined mainly biotic or abiotic parameters in these systems.

Human modifications of the carbon and nitrogen cycles are the subject of intense study due to linkages to the global climate system. While not as well studied, human modifications of the global phosphorus cycle through activities such as mining for phosphate fertilizers may be equally as dramatic (Smil, 2000). Improving our understanding of phosphorus transformations within and among ecosystems will require a better understanding of microbial transformations of organic P compounds. Our studies suggest that the potential for mineralization of IP varies among habitats. Freshwater marshes contain an abundant and diverse community of bacteria that can use one of the most important forms of organic phosphorus, and thus may serve as an important hotspot for P transformations at the landscape scale.

Literature Cited

Fredrickson, J.K. and Balkwill, D.L., 1998. Sampling and enumeration techniques. In: R.S. Burlage, R. Atlas, D. Stahl, G. Geesey and G. Saylor (Editors), Techniques in Microbial Ecology. Oxford University Press, New York, pp. 239-254.

Kerovuo, J. 2000. A novel phytase from Bacillus. Characterization and production of the enzyme. Dissertation Thesis. University of Helsinki.

Lanzetta, P.A., Alvarez, L.J., Reinach, P.S. and Candia, O.A., 1979. An improved assay for nanomole amounts of inorganic phosphate. Analytical Biochemistry, 100: 95-97.

Richardson, A.E., Hadobas, P.A., Hayes, J.E., O'Hara, C.P. and Simpson, R.J., 2001. Utilization of phosphorus by pasture plants supplied with myo-inositol hexaphosphate is enhanced by the presence of soil micro-organisms. Plant and Soil, 229: 47-56.

Rose, T.M., Henikoff, J.G. and Henikoff, S., 2003. CODEHOP (COnsensus-DEgenerate Hybrid Oligonuclueotide Primer) PCR primer design. Nucleic Acids Research, 31: 3763-3766.

Sekar, R. et al., 2003. An improved protocol for quantification of freshwater Actinobacteria by fluorescence in situ hybridization. Applied and Environmental Microbiology, 69: 2928-2935.

Singleton, D.R., Furlong, M.A., Rathbun, S.L. and Whitman, W.B., 2001. Quantitative comparisons of 16S rRNA gene sequence libraries from environmental samples. Applied and Environmental Microbiology, 67: 4374-4376.

Smil, V., 2000. Phosphorus in the environment: natural flows and human interfaces. Ann. Rev. Energy Environ., 25: 53-88.

Suzumura, M. and Kamatani, A., 1995a. Mineralization of inositol hexaphosphate in aerobic and anaerobic marine sediments: implications for the phosphorus cycle. Geochimica et Cosmochimica Acta, 59: 1021-1026.

Suzumura, M. and Kamatani, A., 1995b. Origin and distribution of inositol hexaphosphate in estuarine and coastal sediments. Limnol. Oceanogr., 40: 1254-1261.

Teal, J.M. and Howes, B.L., 1996. Interannual variability of a salt-marsh ecosystem. Limnol. Oceanogr., 4: 802-809.

Turner, B.L., Paphazy, M.J., Haygarth, P.M. and McKelvie, I.D., 2002. Inositol phosphates in the environment. Phil. Trans. R. Soc. Lond. B, 357: 449-469.

Wintzingerode, F., Gobel, U.B. and Stackebrandt, E., 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiology Reviews, 21: 213-229.

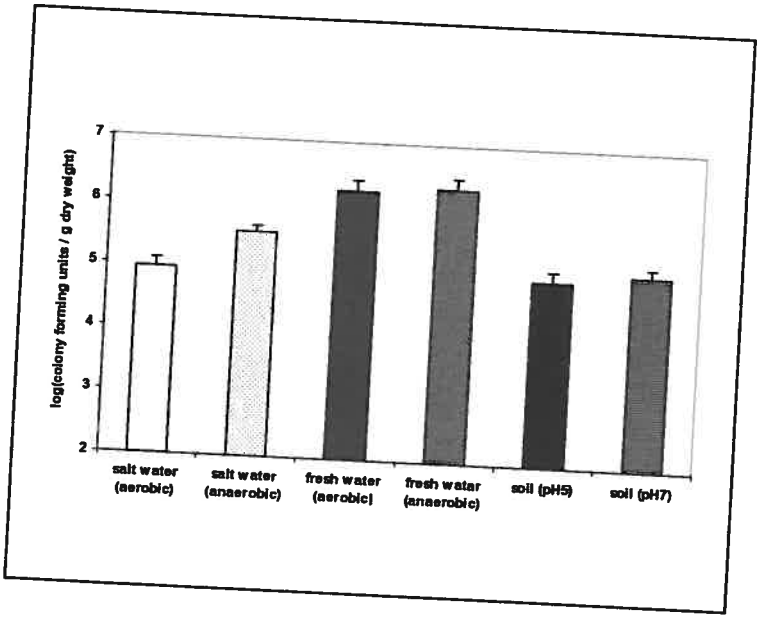

Figure 1. Abundance of colony forming units of bacteria capable of using IP as a sole P source isolated from different environments and culturing conditions (mean ± SE, N=3).

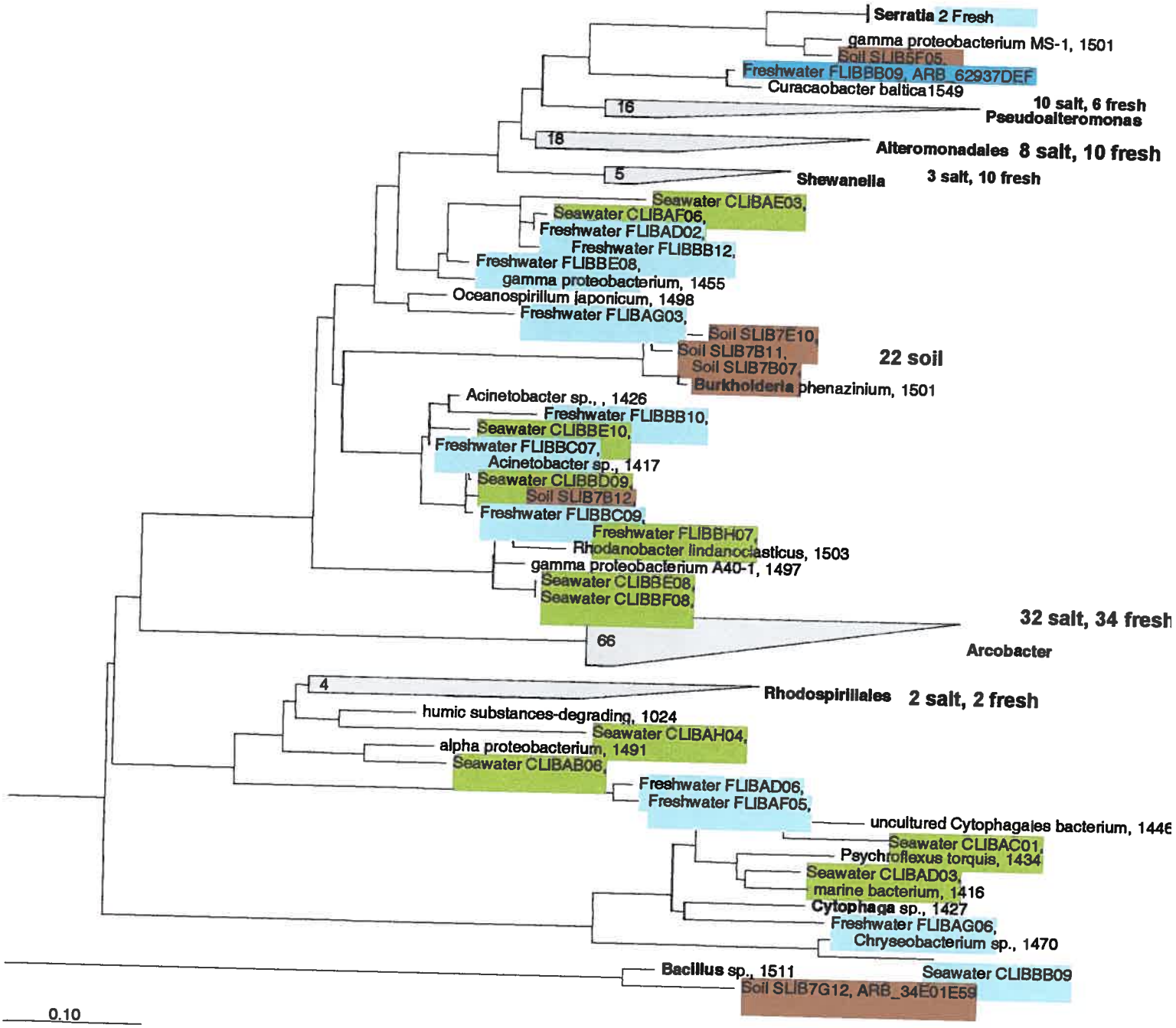

Figure 2. Phylogenetic placement of 205 clones representing cultured IP utilizing bacteria from forest soils (brown), freshwater marsh(blue), and saltwater sediment environment (green). Representatives within collapsed groups are indicated by numbers to the left.

Table 1. rRNA Probes for Fluorescent In Situ Hybridization for Eubacteria

Name	Sequence 5'→ 3'	Target
Eubacteria I	GCTGCCTCCCGTAGGAGT	most bacteria
Eubacteria II	GCAGCCACCCGTAGGTGT	Planctomycetales
Eubacteria III	GCTGCCACCCGTAGGTGT	Verrucomicrobiales

Table 2. Degenerate primers generated for amplification of bacterial 3-phytase

Name	Sequence 5'→ 3'	Degeneracy
Phy1A3 F	GGGCGACGCCATGgaygayccngc	16
Phy2B3 F	CCACCGGCAAGCTGaayaaygtnga	16
Phy3D5 R	CGTTGAACTTCCAGATGCCCwyrtcytcytc	32
Phy4D5 R	TGGGTCACGTCGATGccrtcngtntc	32

Table 3. Pairwise comparisons of species richness of IP using bacteria among forest soils, freshwater marshes, and saltwater marshes (δC). Columns contain X values and rows contain Y values in the comparison of community whether community X differs from Y. The values presented are the observed coverage statistic calculated by LIBSHUFF and p-values are in parentheses.

	Forest soil (N=35)	Freshwater Sediments (N=85)	Saltwater Sediments (N=89)
Forest soil	---	10.68 (0.001)	10.36 (0.001)
Freshwater sediments	10.22 (0.001)	---	0.02 (p=0.495)
Saltwater sediments	11.05 (0.001)	0.061 (0.029)	---

Table 4. Comparison of richness of isolates from aerobic or anaerobic culturing conditions in freshwater and salt water marshes. Sample sizes are in parentheses.

	Culture Condition	Coverage (p-value)	Culture Condition	Coverage (p-value)
Fresh water	aerobic (N=42) anaerobic (N=43)	0.608 (0.001)	anaerobic aerobic	2.024 (0.001)
Salt water	aerobic (N=42) anaerobic (N=43)	5.931 (0.001)	anaerobic aerobic	3.615 (0.001)

Appendix

Medium for enrichment of IP-utilizing microorganisms from soil (pH 5), modified fro 1 Richardson and Hadobas, 1997)

For Soil pH 5 Agar Plates

Steps	Reagents	IP plates	+Pi Plates	No Pi plates
	Final volume	1000 ml	250 ml	250 ml
Add to washed agar with stir bar	Agar	20 g	5 g	5 g
	Glucose (40% w/v)	5 ml	1.25 ml	1.25 ml
	$(NH_4)2SO_4$	1 g	0.25 g	0.25 g
	$MgSO_4*7H_20$	0.1 g	0.025 g	0.025 g
	$CaCl_2*2H_20$	0.1 g	0.025 g	0.025 g
	0.1 M FeNa-EDTA			
	KCl	7.0 g	1.75 g	1.75 g
Bring to volume and autoclave		900 ml H_2O	225 ml H_2O	225 ml H_2O
Add to autoclaved, cooled medium	Complete trace element	1 ml	0.25 ml	0.25 ml
	Cyclohexamide (0.021g/ml)	2.38 ml	0.60 ml	0.60 ml
	0.1 g ml^{-1} Na-IHP (phytic acid)	.154 ml		
	1 M K_2HPO_4		.025 ml	

Add membrane filter-sterilized Na-IHP (10 g / L) to the cooled, autoclaved media. The phytic acid solution (0.1 g mL^{-1}) were adjusted to pH 5 (to prevent precipitation), then filter sterilized prior to addition (i.e. 10 g / 100 ml).

This yields a final concentration of 0.065 M P-phytase and Pi (w/ 50 μg/ ml Cyclohexamide).

For Freshwater pH 7 Agar Plates

Steps	Reagents	IP plates	+Pi Plates	No Pi plates
	Final volume	2000 ml	500 ml	500 ml
Add to washed agar with stir bar	Agar	40 g	10 g	10 g
Bring to volume and autoclave		1700 ml H_2O	450 ml H_2O	450 ml H_2O
Add to autoclaved, cooled medium	40X M	50 ml	12.5 ml	12.5 ml
	Glucose (40%w/v)	10 ml	2.5 ml	2.5 ml
	K2SO4 0.276 M	2 ml	0.5 ml	0.5 ml
	MgCl 0.528 M	2 ml	0.5 ml	0.5 ml
	Cyclohexamide (0.021 g / ml filtersterilized stock)	4.76 ml	1.2 ml	1.2 ml
	0.1 g ml^{-1} Na-IHP (phytic acid)	0.31 ml		
	1 M K_2HPO_4		.05 ml	

Add membrane filter-sterilized Na-IHP (10 g / L) to the cooled, autoclaved media. The phytic acid solution (0.1 g mL^{-1}) was added then filter sterilized prior to addition (i.e. 10 g / 100 ml).

This yields a final concentration of 0.065 M P-phytase and Pi (w/ 50 μg/ ml Cyclohexamide).

For Saltwater pH 7 Agar Plates

Steps	Reagents	IP plates	+Pi Plates	No Pi plates
	Final volume	1500 ml	500 ml	500 ml
Add to washed agar with stir bar	Agar	30 g	10 g	10 g
Bring to volume and autoclave		1100 ml H_2O	350 ml H_2O	350 ml H_2O
Add to autoclaved, cooled medium	40X M	37.5 ml	12.5 ml	12.5 ml
	K_2SO_4 0.276 M	1.5 ml	0.5 ml	0.5 ml
	Glucose (40 % w/v)	7.5 ml	2.5 ml	2.5 ml
	MgCl 0.528 M	1.5 ml	0.5 ml	0.5 ml
	4 M NaCl w/ 100 M Mg2SO4	150 ml	50 ml	50 ml
	Cyclohexamide (0.021g/ml)	3.57 ml	1.2 ml	1.2 ml
	0.1 g ml^{-1} Na-IHP (phytic acid)	0.154 ml		
	1 M K_2HPO_4		.025 ml	

Add membrane filter-sterilized Na-IHP (10 g / L) to the cooled, autoclaved media. The phytic acid solution (0.1 g mL^{-1}) was added then filter sterilized prior to addition (i.e. 10 g / 100 ml).

This yields a final concentration of 0.065 M P-phytase and Pi (w/ 50 μg/ ml Cyclohexamide).

For Soil pH 5 Broth for Dilutions

Steps	Reagents	IP plates	No Pi plates
	Final volume	75 ml	75 ml
	Glucose (40% w/v)	0.75 ml	0.75ml
	$(NH_4)2SO_4$	0.15 g	0.15g
	$MgSO_4*7H_20$	0.015 g	0.015 g
	$CaCl_2*2H_20$	0.015 g	0.015 g
	KCl	1.05 g	1.05 g
Bring to volume and autoclave		57 ml H_2O	57 ml H_2O
Add to autoclaved, cooled medium	Complete trace element	0.15 ml	0.15 ml
	Cyclohexamide (0.021g/ml)	0.357 ml	0.357 ml
	0.1 g ml^{-1} Na-IHP (phytic acid)	0.023 ml	
	1 M K_2HPO_4		

Add membrane filter-sterilized Na-IHP (10 g / L) to the cooled, autoclaved media. The phytic acid solution (0.1 g mL^{-1}) were adjusted to pH 5 (to prevent precipitation), then filter sterilized prior to addition (i.e. 10 g / 100 ml).

This yields a final concentration of 0.065 M P-phytase and Pi.

For Freshwater pH 7 Broth for Dilution

Steps	Reagents	IP plates	No Pi plates
	Final volume	150 ml	150 ml
Bring to volume and autoclave		130 ml H_2O	144 ml H_2O
Add to autoclaved, cooled medium	40X M	3.75 ml	3.75 ml
	Glucose (40%w/v)	0.75 ml	0.75 ml
	K2SO4 0.276 M	0.15 ml	0.15 ml
	MgCl 0.528 M	0.15ml	0.15 ml
	Cyclohexamide (0.021 g / ml filtersterilized stock)	0.36 ml	0.36 ml
	0.1 g ml^{-1} Na-IHP (phytic acid)	0.046 ml	
	1 M K_2HPO_4		

Add membrane filter-sterilized Na-IHP (10 g / L) to the cooled, autoclaved media. The phytic acid solution (0.1 g mL^{-1}) was added then filter sterilized prior to addition (i.e. 10 g / 100 ml).

This yields a final concentration of 0.065 M P-phytase and Pi.

For Saltwater pH 7 Broth for Dilution

Steps	Reagents	IP plates	No Pi plates
	Final volume	75 ml	75 ml
Bring to volume and autoclave		57 ml H_2O	65 ml H_2O
Add to autoclaved, cooled medium	40X M	1.875 ml	1.875 ml
	K_2SO_4 0.276 M	0.075 ml	0.075 ml
	Glucose (40 % w/v)	0.075 ml	0.075 ml
	MgCl 0.528 M	3.75 ml	0.375 ml
	4 M NaCl w/ 100 M Mg2SO4	7.5 ml	7.5 ml
	Cyclohexamide (0.021g/ml)	0.1785 ml	0.1785 ml
	0.1 g ml^{-1} Na-IHP (phytic acid)	0.023 ml	
	1 M K_2HPO_4		

Add membrane filter-sterilized Na-IHP (10 g / L) to the cooled, autoclaved media. The phytic acid solution (0.1 g mL^{-1}) was added then filter sterilized prior to addition (i.e. 10 g / 100 ml).

This yields a final concentration of 0.065 M P-phytate and Pi.