

# Isolation and assessment of phytate-hydrolysing bacteria from the DelMarVa Peninsula

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## Summary

The Delaware–Maryland–Virginia (DelMarVa) Peninsula, flanking one side of the Chesapeake Bay, is home to a substantial broiler chicken industry. As such, it produces a significant amount of manure that is typically composted and spread onto local croplands as a fertilizer. Phytate (*myo* inositol hexakisphosphate), the major form of organic phosphorus in the manure, can be hydrolysed by microorganisms to produce orthophosphate. Orthophosphate is a eutrophication agent which can lead to algal blooms, hypoxia and fish kills in the Chesapeake Bay and its tributaries. This transect study reveals a subpopulation of heterotrophic, thiosulfate-utilizing bacteria that can degrade phytate within the watershed as well as its receiving water sediment. Aerobic isolates were typical soil bacteria, e.g. *Pseudomonas*, *Bacillus* and *Arthrobacter* species, as well as a less common *Staphylococcus* inhabitant. *Bacillus pumilus*, *Staphylococcus equorum*, *Arthrobacter bergei* and *Pseudomonas marginalis* strains have not been previously described as phytate-degrading. Each site along the transect – from manure pile to receiving sediment – was host to a population of bacteria that can degrade phytate and hence, each is a possible non-point source of orthophosphate pollution. Each new isolate could provide an enzyme additive for monogastric feed, thus reducing the impact of excessive phytate load on the environment.

## Introduction

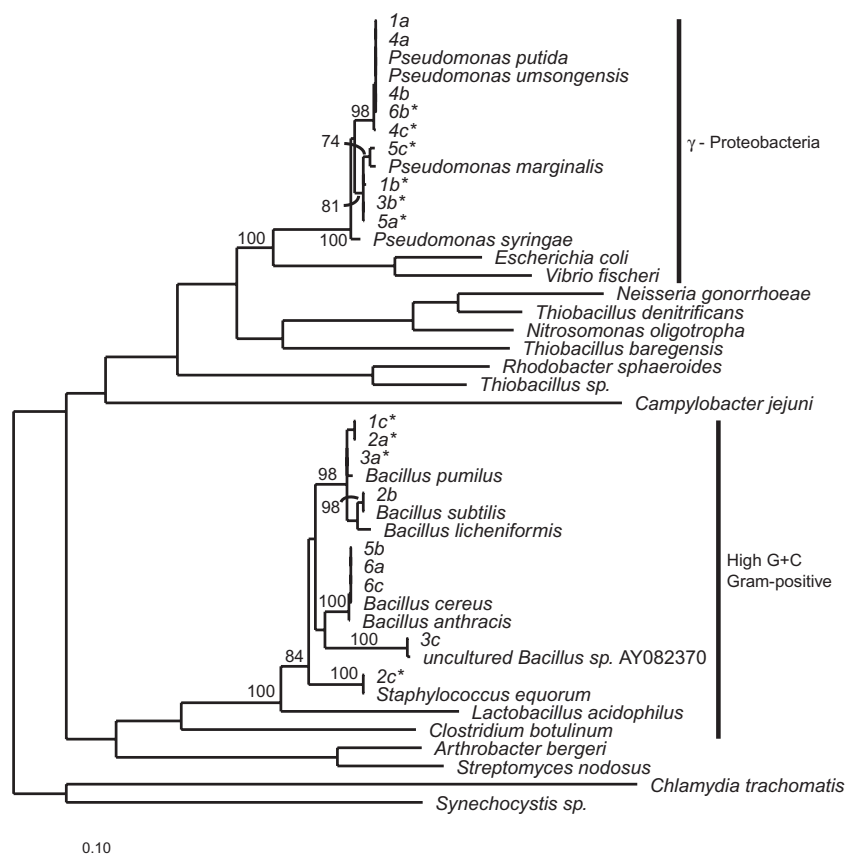
Phytate – *myo* inositol hexakisphosphate – is the main storage form of phosphorus in plants, accounting for 70–80% of the total phosphorus in the seeds of cereal and legume crops (Lott *et al.*, 2000). Intensive agricultural

operations, such as pig and poultry feedlots, use grain as the primary source of macro- and micronutrients. Monogastric animals, however, are unable to degrade phytate (Harland and Morris, 1995; Whittemore, 1995) and it is subsequently excreted in the manure (Whittemore, 1995; Williams *et al.*, 1999; Turner and Leytem, 2004). Additionally, passage through the animal's gastrointestinal tract leads directly to nutritional problems. For example, complexation of the compound with metals such as iron can lead to anaemia (Harland and Morris, 1995), and complexation with proteins can inhibit protein digestion (Rackis, 1974; Erdman, 1979; Maga, 1982)

The manure from intensive agricultural operations is commonly composted and then used as a crop fertilizer. On the Delaware–Maryland–Virginia (DelMarVa) Peninsula, an overapplication of phosphorus from poultry farms is typical (Sharpley, 2000; Sims *et al.*, 2000; Boesch *et al.*, 2001) and the phytate in the manure (Turner and Leytem, 2004) is not directly bioavailable to most plants (Jackman and Black, 1951; Findenegg and Nelemans, 1993; Cakmak *et al.*, 1999; Richardson *et al.*, 2000). Excess phosphorus loading from these non-point pollution sources into freshwater bodies via erosion and surface run-off causes eutrophication. Algal blooms (some harmful), hypoxia, occasional anoxia and catastrophic fish kills are all hallmarks of such excess nutrient addition (Burkholder and Glasgow, 1997; Sharpley, 2000; Sims *et al.*, 2000; Boesch *et al.*, 2001).

The hydrolysis of phytate to orthophosphate and lower substituted inositol phosphates is achieved enzymatically with phytase. Organisms producing phytase have been obtained from such diverse locations as soil (Cosgrove *et al.*, 1970; Richardson and Hadobas, 1997), cattle rumen (Lan *et al.*, 2002), cattle shed floor (Kim *et al.*, 2002), soybean mash (Choi *et al.*, 2001), seawater (Kim *et al.*, 2003), culture collection repositories (Berka *et al.*, 1998; Casey and Walsh, 2004) and plant seeds (Nakano *et al.*, 2000; Greiner, 2004; Greiner and Egli, 2003), suggesting that the ability to degrade phytate might be widely distributed in a variety of ecosystems. Known phytate-degrading organisms include aerobic bacteria [e.g. *Pseudomonas* spp. (Richardson and Hadobas, 1997; Kim *et al.*, 2002), *Bacillus subtilis* (Shimizu, 1992) and *Klebsiella* spp. (Greiner *et al.*, 1997)], anaerobic bacteria [e.g. *Escherichia coli* (Greiner *et al.*, 1993) and *Mitsuokella* spp. (Lan *et al.*, 2002)], fungi [e.g. *Aspergillus*

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**Fig. 1.** Phylogenetic analysis of DelMarVa isolates based on 16S rDNA sequences. The tree was constructed using the ARB software package to align sequences of 1087 positions that were then analysed using PAUP\* to infer a maximum likelihood phylogeny using a Hasegawa–Kishino–Yano DNA substitution model including invariant sites and gamma-distribution rate variation. Neighbour-joining trees from 10 000 bootstrap data sets provide the support values. Isolate number indicates original source of isolate; letter indicates isolate identifier. Phytate-degrading strains are indicated by an asterisk.

spp. (Ullah, 1988; Shimizu, 1992) and *Penicillium* spp. (Tseng *et al.*, 2000)] and plants [e.g. barley (Greiner and Egli, 2003) and wheat (Nakano *et al.*, 2000; Greiner and Egli, 2003)]. However, no study has investigated the prevalence of phytate-degrading organisms across a spatial gradient on monogastric farms, where manure laden with phytate is abundant.

In this study, a spatial transect at a poultry manure-enriched region in the Chesapeake Bay provides samples from which phytate-degrading, aerobic bacteria have been isolated. *Pseudomonad*, *Arthrobacter*, *Staphylococcus* and *Bacillus* species were identified and their phytate-degrading activity was assessed.

## Results

### Enrichment and isolation

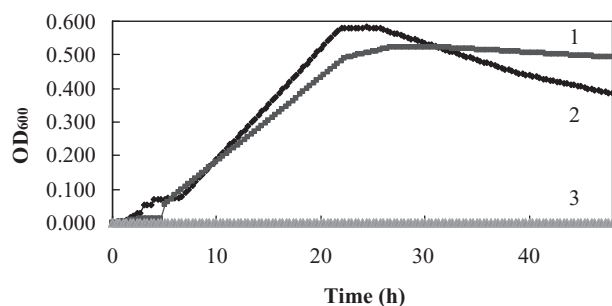
Samples collected from each site [1 – chicken shed floor; 2 – from the culvert next to chicken shed (composite sediment/water); 3 – manure composting pile; 4 – settling pond (composite sediment/water); 5 – Pocomoke River site A (composite sediment/water); and 6 – Pocomoke River site B (composite sediment/water)] were initially enriched on heterotrophic, thiosulfate agar plates with orthophosphate as a source of phosphorus (M1). Forty-

eight isolates were purified by streaking to isolation. Isolates from the original enrichment plate were chosen based on their colony colour and morphology differences (relative to each other). The purified isolates maintained their original colour and morphology with each subsequent subculture. Three isolates were chosen randomly from each sampling location and their identity, phylogeny and phytate-degrading behaviour were explored.

### Identification and phylogenetic context of phytate-utilizing isolates

After DNA extraction, each isolate responded to universal 16S rDNA probes. Phylogenetic analysis of the sequenced amplicons provided taxonomic information for each strain. A phylogenetic tree bearing each isolate is shown in Fig. 1. The first number of each isolate represents the site number from which the isolate was first obtained and the letter is an identification code. Isolates from the genera *Bacillus*, *Pseudomonas*, *Staphylococcus* and *Arthrobacter* were identified.

Primers based on published *Bacillus* species phytase genes (Kerovuo *et al.*, 1998; Tye *et al.*, 2002) were developed and used to interrogate the *Bacillus* species isolated from the DelMarVa Peninsula. Positive controls were



**Fig. 2.** Representative growth curve for isolate 1-c (*Pseudomonas* sp.) on different sulfur sources in M2 medium over 48 h. Series 1 is growth on thiosulfate, series 2 is growth on sulfate and series 3 is growth on tetrathionate.

successful, but no fragments were amplified from any isolate using these primers through the polymerase chain reaction.

#### Sample screening

The 48 isolates were screened via a replica plate technique for their potential to degrade phytate. Seventy per cent of the isolates were capable of using M2 (phytate as sole source of P) for growth. For this plate-based assay, Ca-phytate, a white precipitate, was the only source of phosphorus. Supplementary evidence in the form of zones of clearing on the plates immediately adjacent to the biomass indicated utilization of the Ca-phytate by the isolate via extracellular or outer membrane-bound enzyme(s). The addition of an acid indicator (methyl red) showed that this zone of clearing was acidic in nature. Ten (69%) of the 18 isolates chosen for 16S rRNA determination were able to grow and produce a zone of clearing on M2, indicating potential phytate-degrading ability. Phytate utilization by the 10 isolates was further supported by their growth in liquid culture (M2) at 28°C in scrupulously clean,

acid-washed glassware and via the use of phosphate-free media components. Phytate-degrading strains are indicated in Fig. 1 via an asterisk. Negative controls showed no significant growth for isolates grown on media without any phytate present.

Replica plates and liquid cultures were used to probe sulfur use by the isolates. For those isolates that were designated potential phytate degraders, replica plates with fourfold isolate representation showed growth on sulfate but not on tetrathionate after a 24 h incubation at 28°C. This result was confirmed in liquid culture as is shown for a representative phytate-utilizing isolate in Fig. 2.

#### Phytase assessment

Each isolate was tested for phytate-degrading activity. Liquid cultures were grown for 21 h (stationary phase) in M2 at 28°C. An *E. coli* (known phytate degrader) culture grown to stationary phase in Luria–Bertani broth was always grown in tandem as a positive control. Growth in medium M1, where phosphate is present, produced an overwhelming absorbance signal with the molybdate assay, making the detection of phytase activity difficult. Thus, all results presented here (Table 1) represent those of pure cultures grown in the phosphate-free, phytate medium M2.

After 21 h, culture supernatants were probed for the presence of orthophosphate. Controls with and without inocula showed negligible levels of phosphate in the medium thus, any phosphate measured in the supernate was the result of bacterial activity, most of which can be attributed to the hydrolysis of phytate. Supernate concentrations of orthophosphate ranged from 41  $\mu\text{mol ml}^{-1}$  to 105  $\mu\text{mol ml}^{-1}$ . Of the three highest liberators, two were phylogenetically affiliated with *Pseudomonas marginalis* (3-b: 93  $\mu\text{mol ml}^{-1}$  and 5-c: 104  $\mu\text{mol ml}^{-1}$ ) and one with *Bacillus pumilus* (1-c: 105  $\mu\text{mol ml}^{-1}$ ), even though these

**Table 1.** Phytase activity of DelMarVa isolates: phosphate released by each culture after 21 h as well as the orthophosphate released by supernate enzymes incubated for 7 h.

| Isolate          | Species                        | Supernate $\text{P}_i^a$ ( $\mu\text{mol ml}^{-1}$ ) | Enzyme activity <sup>b</sup> ( $\mu\text{mol ml}^{-1} \text{min}^{-1}$ ) | Phytase localization <sup>c</sup> |
|------------------|--------------------------------|--|--|-----------------------------------|
| 1-b <sup>d</sup> | <i>Pseudomonas marginalis</i>  | 70   | 0.04   | EX                                |
| 1-c <sup>d</sup> | <i>Bacillus pumilus</i>        | 105  | n.m.   | CA                                |
| 2-a <sup>d</sup> | <i>Bacillus pumilus</i>        | 56   | 0.04   | EX                                |
| 2-c <sup>d</sup> | <i>Staphylococcus equorum</i>  | 41   | 0.04   | EX                                |
| 3-a <sup>d</sup> | <i>Bacillus pumilus</i>        | 67   | 0.06   | EX                                |
| 3-b <sup>d</sup> | <i>Pseudomonas marginalis</i>  | 93   | 0.03   | EX                                |
| 4-c <sup>d</sup> | <i>Pseudomonas umsongensis</i> | 61   | n.m.   | CA                                |
| 5-a <sup>d</sup> | <i>Pseudomonas marginalis</i>  | 52   | n.m.   | CA                                |
| 5-c <sup>d</sup> | <i>Pseudomonas marginalis</i>  | 104  | n.m.   | CA                                |
| 6-b <sup>d</sup> | <i>Pseudomonas umsongensis</i> | 62   | 0.05   | EX                                |

a. Orthophosphate release after 21 h incubation with crude culture supernatant.

b. Orthophosphate released from culture supernate activity at 37°C.

c. Enzyme activity was either extracellular (EX) or cell associated (CA).

d. Phytate-degrading strain.

n.m., not measurable.

isolates did not have the highest optical densities at 600 nm after 21 h (data not shown). The theoretical maximum orthophosphate concentration derived from phytate is 190  $\mu\text{mol ml}^{-1}$ .

## Discussion

We isolated aerobic bacteria from diverse sampling locations along a hypothetical phytate fate pathway at an active poultry operation on the DelMarVa Peninsula. A subpopulation of bacteria that seemed particularly adept at degrading phytate was isolated on plates using an enrichment combination of thiosulfate, glucose and orthophosphate. On average, 70% of these isolated bacteria were able to grow on phytate and produce an acidic zone of clearing on plates containing calcium phytate as the sole source of phosphorus (Richardson and Hadobas, 1997; Hill and Richardson, 2006).

The *Bacillus* strains isolated from the DelMarVa Peninsula can be segregated into four clades based on their 16S rDNA sequences: *Bacillus cereus/anthracis*, *B. subtilis/lichiniformis*, *B. pumilus*, and one close to an uncultured *Bacillus* species. The isolated *B. pumilus* species all produced a positive response to the plate-clearing assay; however, the *B. subtilis* clade isolate did not grow on a calcium phytate agar plate, nor did the *B. anthracis* isolates or the isolate aligned with an uncultured strain. This lack of growth could be a reflection of the isolates' inability to degrade phytate or perhaps, merely an indication of unfavourable conditions for phytase production. Several phytate-degrading *Bacillus* species have been published previously, including *Bacillus amyloliquefaciens* (Kerovuo *et al.*, 1998), *Bacillus* sp. DS11 (Kim *et al.*, 1998) and *B. subtilis* strains (Powar and Jagannathan, 1982; Shimizu, 1992), with each producing a positive response to the plate-clearing as well as an enzyme activity assay. Growth on plates is usually easier than in a liquid medium for phytate-degrading bacteria (Choi *et al.*, 2001; Hill and Richardson, 2006), hence, no further assessment was conducted on strains that could not grow on plate systems. *B. pumilus* strains were probed with phytase gene primers known to work on other phytase-producing *Bacillus* species (Kerovuo *et al.*, 1998; Tye *et al.*, 2002). These strains did not produce an amplification product; however, they are likely to host a version of the known beta propeller phytase family.

Nine pseudomonads were isolated in this study, with five showing phytate-degrading potential via growth and production of a zone of clearing on agar media containing calcium phytate. *Pseudomonad* production of phytase has been described previously. For example, Richardson and Hadobas (1997) identified four *Pseudomonas* isolates, two that cluster in the *Pseudomonas putida* clade and two that cluster in the *Pseudomonas mendocina*

clade. A *Pseudomonas fragi* (strain Y9451) isolate has been identified as producing a phytase enzyme (In *et al.*, 2004), and Kim and colleagues (2003) also identified a pseudomonad, but not to the species level. The isolates from this survey cluster in two clades, *P. putida/umsongensis* and *P. marginalis*. Three out of five in the former and all of the latter showed phytate-degrading potential. The lack of activity in two of five *Pseudomonad* isolates of the *P. putida* clade could be due to a loss of trait in the strain, or perhaps the culture conditions used in this study were not effective in inducing the production of phytase in these particular isolates. It is unclear at this point whether phytate hydrolysis is a common trait within the genus *Pseudomonas*.

Bacteria from the genera *Staphylococcus* and *Arthrobacter* have not been shown previously to degrade phytate. Species of the genus *Staphylococcus* are Gram-positive, facultative anaerobes often associated with warm-blooded animals in a pathogenic context. The species in this survey correlates most closely with *Staphylococcus equorum*, which is normally associated with food products like meat and dairy (Ghosh *et al.*, 2006). It is the first *Staphylococcus* isolate reported to show definitive evidence of phytate-degrading activity. Species of the genus *Arthrobacter* are Gram-positive, aerobic bacteria widely distributed in soil environments. The species isolated in this survey has *Arthrobacter bergei* as the nearest relative based on 16S rDNA sequence identity. While amplification of the 16S rRNA gene beyond 500 bp was not possible for the *Arthrobacter* isolate under the primer conditions applied in this study, the high sequence identity is significant enough to confidently place the isolate in the *Arthrobacter* clade. *Arthrobacter* was shown to produce low levels of phytase activity in the culture supernate after 21 h of growth on phytate (J.E. Hill, unpublished). However, most of the activity appeared locked in the cell debris and thus it could not be consistently quantified. This study presents the first *Arthrobacter* isolate reported to show phytate-degrading activity.

For the phytate-degrading bacteria isolated in this study, orthophosphate liberation was generated in most cases by an extracellular phytase enzyme. This was confirmed through the measurements of crude and semi-purified cell extracts. Higher yields of orthophosphate from the action of a phytate-degrading enzyme may be possible after more purification, which would eliminate possible suppression of enzyme activity caused by orthophosphate in the medium (Touati and Danchin, 1987; Greiner *et al.*, 1993) as well as decrease the presence of organic material, which is known to bind both phytate (Borie *et al.*, 1989; Nanny and Minear, 1994) and molybdate (Turner *et al.*, 2006). Especially in the cases of cell-bound activity, a qualitative confirmation – through the observation of a substantial yellow-coloured precipitate

collected upon sample centrifugation – indicates that a greater activity was present than was reflected in the absorbance reading. The sonication of whole-cell culture suspensions with isolates known to have extracellular activity produced similar results, confirming the likely production of cell debris-associated, molybdate-complexed phosphate.

The significance of metabolism of both thiosulfate and phytate is not yet fully understood; however, some hypotheses from this work can be generated. We have shown here that the cultures isolated from this study will grow on sulfate and thiosulfate, but not on tetrathionate, a polymer of thiosulfate ( $S_2O_6^{2-}$ ). In addition we have shown that growth on thiosulfate leads to the production of acid. Coupled with some preliminary results from the amplification of the *soxB* gene (J.E. Hill, unpublished) from several isolates, this work suggests that the isolates are utilizing the *Paracoccus* pathway, one of the two major pathways for the oxidation of thiosulfate (Kelly *et al.*, 1997). It could be argued that the increase in energy generated by use of the *Paracoccus* pathway, along with the production of acid, makes phytate more soluble and therefore more bioavailable (Evans and Martin, 1987; 1988a,b; Turner *et al.*, 2002). This could confer a selective advantage on organisms in microaerophilic niches in a variety of soil and terrestrial environments. The link between organic phosphorus cycling and sulfur oxidation warrants further research.

This study reveals that in every sample, there are common aerobic bacteria that can degrade phytate. Thiosulfate oxidation by these organisms – whether purposeful or incidental – will produce acidic conditions, which are likely to enhance the hydrolysis of phytate by organisms in that niche. The subsequent production of orthophosphate by these cell-bound or extracellular enzymes could therefore be creating multiple sources of relatively mobile, non-point phosphorus pollutant – orthophosphate. This suggests that point-source pollution control strategies, such as phytase addition to animal feed, provide a sensible approach to managing phosphate contamination. The release of orthophosphate by manure and soil populations also hints that, given the right circumstances, native bacterial communities can degrade phytate. The resulting release of orthophosphate, which is more bioavailable to plant communities, could thus enhance the benefits of manure-derived fertilizer.

## Experimental procedures

### Enrichment inocula source

Composite samples were collected aseptically from six locations on the DelMarVa Peninsula, spanning the spatial gradient from a commercial chicken shed to the closest, down-gradient receiving water body (Pocomoke River, MD),

relative to the shed. Specifically, the locations were as follows: 1 – chicken shed floor; 2 – from the culvert next to chicken shed (composite sediment/water); 3 – manure composting pile; 4 – settling pond (composite sediment/water); 5 – Pocomoke River site A (composite sediment/water); and 6 – Pocomoke River site B (composite sediment/water). Samples were stored at 0°C for 24–36 h before plating.

### Culture media

Enrichment media varying in phosphorus, carbon and sulfur source were used to culture isolates from each sampling location. The combination producing the highest proportion of isolates exhibiting potential phytate hydrolysis was found using an enrichment medium containing orthophosphate, thiosulfate and glucose. These organisms and conditions of enrichment were studied further.

Three media recipes were used to isolate bacteria and assess their potential to degrade phytate. Where relevant, all glassware was acid-washed and all medium components were orthophosphate-free. Per litre, each medium contained: 10.0 g of  $Na_2S_2O_3 \cdot 5H_2O$ , 8.0 g of glucose, 1 g of  $MgCl_2 \cdot 6H_2O$ , 0.30 g of  $NH_4Cl$ , 0.04 g of  $FeCl_3 \cdot 6H_2O$  and 1.0 ml of trace element solution. Medium 1 (M1) also contained (per litre) 1.5 g of  $K_2HPO_4$ , 1.5 g of  $KH_2PO_4$  and 0.01 g of biotin. Medium 2 (M2) also contained (per litre) 3.0 g of sodium phytate, 5 g of  $CaCl_2 \cdot 2H_2O$  and 0.01 g of biotin. Filtered (0.22  $\mu m$ ) sodium phytate was added after autoclaving other ingredients. The pH of each solution was adjusted to 7.0 using 25% HCl. Where relevant, 20 g l<sup>-1</sup> agar was added. All media ingredients were purchased from Sigma (ultrapure). Two additional media components, sulfate and tetrathionate, were used to assess the growth of the bacterial isolates on additional sulfur sources. These media were based on M2 with an equimolar substitution used for each sulfur source tested.

### Isolation and purification

Five grams of each DelMarVa sample was suspended in 20 ml of sterile 10 mM KCl and placed on a rotary shaker (5 r.p.m.) at 4°C for 24 h before addition to culture media. A small aliquot (20  $\mu l$ ) of the suspension was spread on an M1 agar plate and incubated at 28°C until growth occurred. Subcultures from the enrichment plate, chosen for their unique colony morphology, were streaked onto fresh agar plates of the same composition. Each subculture was streaked to an individual colony at least three times to ensure purity. Stock cultures were stored in glycerol at -70°C.

### DNA extraction, sequencing and phylogenetic analysis

Pure cultures were grown in M1 broth overnight. DNA extraction from 1 ml of pure culture (approximately  $10^8$  cells ml<sup>-1</sup>) was achieved with Qiagen's DNAeasy Tissue Culture kit (Qiagen). 16S rRNA genes were amplified with universal primers 27f (AGA GTT TGA TCM TGG CTC AG), 516f (TGC CAG CAG CCG CGG TAA), 1046r (GAC AGC CAT GCA VCA CCT) and 1492r (CGG YTA CCT TGT TAC GAC TT) (Weisburg *et al.*, 1991) using Amplitaq® polymerase (Applied

Biosystems). All primer pair combinations were assessed via PCR. Conditions for amplification were 94°C for 2 min; followed by 25 cycles of 94°C (30 s), 54°C (30 s), 72°C (90 s); and finished with 72°C for 5 min. Amplified DNA was purified using the QIAquick PCR Purification Kit (Qiagen). The purified product was then sequenced with the Taq dideoxy terminator cycle sequencing kit (Applied Biosystems) using modified universal primers 27f or 516f and an ABI PRISM® model 310 genetic analyser.

Low-quality terminal 16S rDNA sequence regions were trimmed using CodonCode Aligner. The sequence data were compiled into a comprehensive 16S rRNA gene database (<http://download.arb-home.de>) using the ARB software package (Ludwig *et al.*, 2004). The GenBank accession numbers for the sequences reported in this article are: DQ232731–48. The ARB FastAligner utility aligned the gathered sequences to the comprehensive database alignment, which we then manually adjusted. A well-aligned subset of 1087 sequence positions from the collected data and representative database sequences provided the basis for all phylogenetic analyses. We used PAUP\* version 4.0b10 for UNIX (Swofford, 2003) to infer a maximum likelihood phylogeny using a heuristic search strategy under a Hasegawa–Kishino–Yano DNA substitution model (Hasegawa *et al.*, 1985) that included invariant sites and gamma-distributed rate variation (HKY+I+G). Neighbour-joining trees from 10 000 bootstrap data sets and based on a HKY+I+G distance model provided support values for key nodes in the tree.

#### *Bacillus phytase oligonucleotide probe design*

Oligonucleotide primers specific to the *Bacillus* phytase gene were designed using sequences loaded into Sequencher™ (version 4.5). MacVector (version 7.2.3) was used to design the primers, which were manually checked against the conserved region of sequence space in Sequencher. *In silico* and experimental controls showed appropriate responses. The primer sequences were as follows: b1f: GGA TGG CAG CAG ACG ATG AAT AC; b2r: TTA TTT TCC GCT TCT GTC; b3r: TGT CGG TCA GTT TTC TCG GGT C; b4f: CTC GCA TGC TGT CCG ATC CTT ATC ATT TTT ACC G; k5r: GGC AGA TCT TTT TCC GCT TCT GTC GGT CAG TTC; and k6r: CGT TCA ATT GAG GAG GAA GTA AAA TGA ATC.

#### *Growth and enzyme assays*

Replica plates with fourfold isolate representation were used to interrogate all enrichment isolates. Replica plates made with M1 served as stock cultures. M2 medium was used to assess utilization of phytate as a sole source of phosphorus at 28°C.

A template with all cultures represented in fourfold replication was created on M1 agar plates. Template impregnation onto M2 medium was used to evaluate an isolate's potential to grow on calcium phytate as the sole source of phosphorus at 28°C over 24 h. Liquid assays using M2 medium confirmed utilization of phytate. Stock cultures were prepared overnight and used to inoculate a 96-well plate (5 µl of culture and 250 µl of M3 medium) covered with a breathable, plastic

cover (3M Transparent polyolefin). Measurements of OD<sub>600</sub> (SpectraMax Microplate reader 340PC384) were made every 20 min.

Three isolates from each site were chosen randomly for further investigation. Each experiment described below was conducted using acid-washed glassware and with freshly prepared cultures. Phytate-degrading abilities were ascertained by two methods after 21 h of growth. In each scenario, *E. coli* – a known phytate degrader – was used as a positive control. Crude supernatant was directly assayed for orthophosphate liberation. Separately, crude supernate and cell pellet fractions were refined via ethanol precipitation.

Crude culture supernatant was subjected to three volumes of ethanol (–20°C) on a rotary shaker (5 r.p.m.) for 30 min, followed by overnight incubation at 4°C, and then centrifugation at 4000 *g* for 10 min. The supernate was decanted and the pellet was washed with 1 ml of ethanol and then 1 ml of acetone. The pellet was then dried in a laminar hood for 1 h, then re-suspended in 1 ml of Tris-HCl (pH 7.0) and stored on ice for use in 30 min incubation-based phytate degradation assays (see below).

The culture cell pellet was suspended in lysozyme treatment [2 mM EDTA, 1.2% Triton® X-100, 20 mM Tris-HCl (pH 8) and 20 mg ml<sup>-1</sup> lysozyme] for 30 min at room temperature. The degraded cell pellet was then subject to three volumes of ethanol (–20°C) on a rotary shaker (5 r.p.m.) for 30 min, followed by overnight incubation at 4°C, and then centrifugation at 4000 *g* for 10 min. The supernate was decanted and the pellet was washed with 1 ml of ethanol then 1 ml of acetone. The pellet was then dried in a laminar hood for 1 h. The pellet was then re-suspended in 1 ml of Tris-HCl and stored on ice for use in 30 min phytate-degradation assays.

For each preparation (crude and partially purified by ethanol precipitation), phytate degradation, via orthophosphate evolution, was assessed at 37°C in an acetate (pH 5.5) buffer. Briefly, 200 µl of enzyme preparation was added to 100 µl of buffer in a 1.5 ml microcentrifuge tube. After mixing by vortex, the solution was incubated for 5 min at 37°C. To this mixture, 600 µl of phytate (7.5 mM) was added and then incubated for either 0 h (culture supernate) or 0.5 h (post-ethanol precipitation), at 37°C. A stop reagent consisting of one volume of ammonium vanadate (2.35%), one volume of ammonium molybdate (10%) and two volumes of nitric acid (65%) was added to the solution, followed by incubation at room temperature for 10 min. Each tube was then subjected to centrifugation for 3 min at 14 600 r.p.m. Phosphate liberation was assessed at 415 nm using a spectrophotometer (Bio-Rad SmartSpec 3000). A calibration curve was established over a range of 0–50 µg ml<sup>-1</sup> of inorganic phosphate. Protein concentration was determined using the Bradford method (Bradford, 1976) with a kit from Sigma and HSA as the standard.

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