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2 1 Improved sensitivity, accuracy and prediction provided by a High-Performance Liquid  
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4 2 Chromatography screen for the isolation of phytase-harboring organisms from  
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6 3 environmental samples.  
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10 5 Running title: MINPP activity in soil-dwelling *Acinetobacter*  
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45 19 acid phosphatase; multiple inositol phosphate phosphatase; soil phytate  
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## 1 Summary

2 HPLC methods are shown to be of predictive value for classification of phytase  
3 activity of aggregate microbial communities and pure cultures. Applied in initial  
4 screens, they obviate the problems of 'false-positive' detection arising from impurity  
5 of substrate and imprecision of methodologies that rely on phytate-specific media.  
6 In doing so, they simplify selection of candidates for biotechnological applications.  
7 Combined with 16S sequencing and simple bioinformatics, they reveal diversity of  
8 the histidine phosphatase class of phytases most commonly exploited for  
9 biotechnological use. They reveal contribution of Multiple Inositol Polyphosphate  
10 Phosphatase (MINPP) activity to aggregate soil phytase activity and they identify  
11 *Acinetobacter* spp. as harbouring this prevalent soil phytase activity. Previously,  
12 among bacteria MINPP was described exclusively as an activity of gut commensals.  
13 HPLC methods have also identified, in a facile manner, a known commercially  
14 successful histidine (acid) phosphatase enzyme. The methods described afford  
15 opportunity for isolation of phytases for biotechnological use from other  
16 environments. They reveal the position of attack on phytate by diverse histidine  
17 phosphatases, something that other methods lack.

18

## 19 Introduction

20 There are four forms of phytic acid (inositol hexakisphosphate, InsP<sub>6</sub>) which have  
21 been identified in nature, *myo*-, *neo*-, *scyllo*- and *D-chiro*- that differ in their  
22 stereochemical conformation (Figure S1) and association with metal ions as phytates  
23 in different soils (Turner *et al.*, 2002). Among these, *myo*-inositol hexakisphosphate  
24 (InsP<sub>6</sub>) garners the most attention from plant scientists. It is the principal storage

1 form of phosphorous in plants, seeds and grains representing between 50-85% of  
2 the total phosphate in plants and forming as much as 1-5% of the dry weight in many  
3 seeds, grains and fruits (Raboy & Dickinson, 1993).

4  
5 Monogastric animals such as swine and poultry are fed diets that are largely cereal-  
6 and/or grain-based, but they lack sufficient levels of endogenous phytase, a mixed  
7 group of phosphatases that dephosphorylate phytate (Pandey *et al.*, 2001). The  
8 undigested phytate and other 'higher' inositol phosphates are potent anti-nutrients  
9 by virtue of their ability to interfere with protein digestion and to chelate metal ions  
10 such as calcium, iron, magnesium, manganese and zinc, reducing their bioavailability.  
11 The first commercially produced phytase, *Natuphos*<sup>®</sup>, was released to the market in  
12 1991 to improve the digestibility of grain phytate in the gastrointestinal tract of non-  
13 ruminants (Lei & Porres, 2003). Since then, phytases have become a major sector of  
14 a global enzyme market of estimated value ca. \$5 billion in 2015, with annual growth  
15 estimated at 6-8% from 2016-2020 (Guerrand, 2018).

16  
17 Phytases are commonly separated into four categories,  $\beta$ -propeller phytases  
18 ( $\beta$ PPhy), Purple Acid Phytases (PAPhy), Protein Tyrosine Phytases (PTPs) (Cysteine  
19 phytases) and the Histidine (Acid) Phosphatases (Mullaney & Ullah, 2007). The  
20 histidine (acid) phosphatases also comprise a subclass named Multiple Inositol  
21 Polyphosphate Phosphatases (MINPP) (Cho *et al.*, 2006; Mehta *et al.*, 2006; Haros *et*  
22 *al.*, 2009; Tamayo-Ramos *et al.*, 2012; Stentz *et al.*, 2014). The search for more  
23 effective enzymes - encompassing improved catalytic efficiency, protease-, acid- and  
24 thermo-stability - and cost-effective production has been extended to soil

1 environments where *myo*-, *neo*-, *scyllo*-, and *D-chiro*- forms of phytate represent  
2 substantial, albeit recalcitrant, 'reserves' of organic phosphate (Menezes-Blackburn  
3 *et al.*, 2018). The soil environment encompasses a diverse microflora, with estimates  
4 of 4000-7000 different bacterial genomes per gram of soil (Ranjard *et al.*, 2000).  
5 Consequently, soil has been a target for many phytase isolation efforts (Kumar *et al.*,  
6 2013; Puppala *et al.*, 2019). Characterization of enzymes isolated from different  
7 environments has allowed comprehensive comparative analysis of stability and  
8 activity (Konietzny and Greiner, 2002; Mullaney & Ullah, 2003; Huang *et al.*, 2006),  
9 aiding the development of thermo-stable enzymes for industrial use (Lehmann *et al.*,  
10 2000; Wu *et al.*, 2014). Nevertheless, several technical issues still frustrate efforts to  
11 identify and isolate phytase-producing organisms, and assessment of their  
12 contribution to environmental turnover of organic phosphates, including phytate.  
13  
14 The small fraction of environmental organisms amenable to culture has the  
15 consequence that the biodiversity of phytase producers is grossly underestimated.  
16 Consequently, metagenomic and metaproteomic approaches have supplanted  
17 culture-based approaches for study of the relationship of microbiological diversity  
18 and soil phosphorus (Neal *et al.*, 2017; Yao *et al.*, 2018; Chen *et al.*, 2019).  
19 Alternatively, others have employed amplicon sequencing of functional  
20 phosphatases using *phoD* alkaline phosphatase specific primers (Ragot *et al.*, 2015).  
21 When allied with heterologous expression, metagenomic methods have revealed  
22 novel catalytic diversity among phytate-degraders extending classification beyond  
23 the four canonical classes (Castillo Villamizar *et al.*, 2019a,b) as have more  
24 conventional functional genomic methods (Sarikhani *et al.*, 2019).

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4 2 Irrespective of the method of identification of candidate phytases, whether as  
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6 3 commercial product leads or as contributors to environmental processes, both  
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8 4 culture-independent approaches and their culture-dependent counterparts  
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11 5 commonly rely on informative enzyme assays for characterisation of the reactions  
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13 6 catalysed. One issue with the assay most commonly used, phosphate detection with  
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15 7 reagents such as molybdenum blue/malachite green, is the purity of the phytate  
16  
17 8 substrate. Commercially available phytate is impure (Figure S2A), and often contains  
18  
19 9 substantial mole fractions of lower inositol phosphate and inorganic phosphate  
20  
21 10 impurities (Nagul *et al.*, 2015). Consequently, unless assays follow disappearance of  
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23 11 phytate they risk measurement of pre-existing inorganic phosphate or risk  
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25 12 misidentification of enzymic activity towards 'lower' inositol phosphates. The  
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27 13 literature offers historic precedent: isolates capable of degrading  $\text{InsP}_5$  but not  $\text{InsP}_6$   
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29 14 were identified in a seminal study of phytase isolation (Cosgrove *et al.*, 1970).  
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36 15  
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38 16 The issue of substrate quality is relatively solvable; purification of  $\text{InsP}_6$  is well  
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40 17 described (Cosgrove, 1980; Dorsch *et al.*, 2003; Madsen *et al.*, 2019), but rarely  
41  
42 18 discussed in screening for phytases. "Phytase specific media" (PSM) (Howson &  
43  
44 19 Davis, 1983; Kerovuo *et al.*, 1998) is used widely and relies on formation of clearing  
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46 20 (of phytate precipitate) zones around bacterial colonies. The method suffers a high  
47  
48 21 rate of false-positives, arising from bacterial secretion of low molecular weight  
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50 22 organic acids capable of solubilizing the phytate precipitates (Iyer *et al.*, 2017). This  
51  
52 23 itself highlights another issue with the approach – that it is not suitable for screening  
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54 24 at low pH – a condition for which many commercial enzymes have been optimized.  
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1 While solubilisation may be overcome by a two-step counter-staining test to re-  
2 precipitate acid-solubilised phytate (Bae *et al.*, 1999), re-precipitation does not  
3 indicate to what extent the available phytate has been degraded, since other 'higher'  
4 inositol phosphates can also be re-precipitated. Autoclaving of the medium can also  
5 result in phytate degradation (see Figure S2B,C) and resultant change to the pH of  
6 the media. Overall, clearing zones may not exclusively indicate enzymatic hydrolysis  
7 of phytate in the plate (Fredrikson *et al.*, 2002), while pH limitations of the method  
8 will necessarily be selective of the organisms cultured. There is, therefore,  
9 opportunity for sensitive methodologies that allow characterization of the substrate  
10 and its utilization. Here we adopt the PSM methodology and supplement it with High  
11 Performance Liquid Chromatography (HPLC) to demonstrate a more accurate and  
12 quantitative method allowing screening and isolation of phytase-producing  
13 organisms from environmental samples. We also show how different isolates  
14 produce different inositol phosphate profiles from phytate and extend the analysis  
15 to soil samples supplemented with phytate to follow the activity of aggregate  
16 cohorts of microbes. A schematic diagram of the range of analyses enabled is shown  
17 (Figure S3).

18

## 19 Results and discussion

### 20 Acid-extraction of phytate from PSM plates

21 The PSM plate approach is one of the most commonly used methods for isolation of  
22 phytase-positive organisms from soil, but it is not without the substantial drawbacks  
23 discussed above. Control strains of *Escherichia coli*-pDES17-*Btminpp* harbouring a  
24 plasmid-borne MINPP from *Bacteroides thetaiotaomicron* (Stentz *et al.*, 2014),

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1 *Bacillus subtilis* strain ESKAPE (predicted to contain  $\beta$ PPHy) and *Pseudomonas putida*  
2 J450 (predicted to contain  $\beta$ PPHy) were each streaked onto fresh PSM plates and  
3 allowed to grow over three days at 30 °C. All isolates generated clearing zones  
4 around their biomass on these PSM plates. Cores of agar from 'cleared' and 'non-  
5 cleared, cloudy' zones were extracted with HCl and the inositol phosphate profile  
6 thereof examined by HPLC (Figure 1). While there can be slight differences in the  
7 efficiency of extraction between the cleared and cloudy zones, comparison of  
8 individual peaks within the respective profiles makes evident the different extents  
9 and pathways of phytate degradation by the strains.

10  
11 Figure 1.

12  
13 All profiles from the 'non-cleared' zones show the predominant peak of  $\text{InsP}_6$  with a  
14 retention time of c. 37 minutes and a smaller peak of  $\text{InsP}_5$  [1/3-OH] contaminant  
15 with a retention time of c. 28 minutes, representing approximately 5% of total  
16 inositol phosphate in this 'clean'  $\text{InsP}_6$  substrate. Inorganic phosphate (Pi) elutes with  
17 the solvent front at c. 2.8 min. In respect of the 'cleared' zones, the *B. subtilis* strain  
18 ESKAPE (Figure 1A) showed a small amount of  $\text{InsP}_6$  degradation with a small  
19 increase in  $\text{InsP}_5$  [1/3-OH], and a concomitant increase in Pi. In this experiment  
20 much of the  $\text{InsP}_6$  remained. The  $\text{InsP}_5$  [1/3-OH] peak is the expected product of the  
21 known  $\text{InsP}_6$  D-3-phosphatase activity of the  $\beta$ PPHy (Kerovuo *et al.*, 1998) originally  
22 characterized (Powar & Jagannathan, 1982). The *E. coli*-pDES17-*Btminpp* strain  
23 (Figure 1B) showed considerably more activity, producing multiple peaks of  $\text{InsP}_5$ ,  
24  $\text{InsP}_4$  and  $\text{InsP}_3$  intermediates, characteristic of MINPP enzyme (Haros *et al.*, 2009;

1 Tamayo-Ramos *et al.*, 2012; Stentz *et al.*; 2014). There is also a larger Pi peak.  
2 Finally, the *P. putida* strain (Figure 1C) showed little difference in the profile of  
3 'cleared' vs. 'non-cleared' agar despite the known InsP<sub>6</sub> D-3-phosphatase activity of  
4 other *Pseudomonas* sp. (Cosgrove *et al.*, 1970; Irving and Cosgrove, 1972).

5  
6 Collectively, these comparisons demonstrate that zone clearing without careful  
7 normalization is a poor assay for phytate degradation even of well-characterized  
8 organisms. It does illustrate however that HPLC can be combined with media-based  
9 culture and extraction of agar for testing of phytate degradation to provide high  
10 sensitivity and diagnostic analysis of the likely enzyme activity, by the simple  
11 expedient of observation of the occurrence of InsP peaks not present in 'non-  
12 cleared' regions of agar plates.

#### 13 14 Assay of phytate degradation by mixed population soil cultures

15 Phytate degradation may also be demonstrated with mixed cultures that might  
16 ordinarily be subjected to standard dilution and culture techniques for  
17 discrimination of individual isolates. In Figure 2 we show the result of mixing soil  
18 with minimal medium containing InsP<sub>6</sub> as the sole phosphate source. The soil was  
19 untilled (for the season) agricultural soil from Fakenham, Norfolk, UK, which we used  
20 to first test the technique. In this experiment, this agricultural soil was incubated  
21 with shaking at 30 °C. Degradation of InsP<sub>6</sub> was observed initially on day 3; by day 5  
22 less than 5% of starting InsP<sub>6</sub> remained, consistent with the accumulation of Pi,  
23 which co-elutes with InsP<sub>1</sub> on this column-gradient method. The generation of  
24 multiple inositol phosphate peaks at all stages of dephosphorylation (InsP<sub>5</sub>, InsP<sub>4</sub>,



1 InsP<sub>3</sub> and InsP<sub>2</sub>) probably arises as a consequence of the action of several phytase  
2 enzymes, since the classification of phytases reflects predominant attack in discrete  
3 sequences and predominant accumulation of single InsP<sub>5</sub> and InsP<sub>4</sub> species.

4  
5 Figure 2.

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7 This experiment was repeated on five well-characterised soil or plant-growth  
8 matrices, all of which showed evidence of phytase activity. The first (Figure 3A) is  
9 Levington Compost F2, obtained from the John Innes Centre, Norwich, UK. The  
10 second (Figure 3B) is soil sampled from Church Farm, the field study site of the John  
11 Innes Centre in Bawburgh, Norwich UK. The next three soils were sampled from two  
12 long-term field experiments from Rothamsted Research, Harpenden, UK  
13 (Supplementary information). The first sample (Figure 3C) was obtained from  
14 continuous arable plots growing winter wheat (*Triticum aestivum* L.) of the Highfield  
15 Ley-Arable experiment. Also, from this site, soil was sampled from permanent bare  
16 fallow plots (Figure 3E) that have been maintained crop- and weed-free by regular  
17 tilling for over 50 years. The  $\beta$ PPHy genes in both these soils have been characterized  
18 by shotgun metagenomics (Neal *et al.*, 2017). The gene sequences identified show  
19 homology to genes identified in *Bacillus*, *Paenibacillus*, *Alteromonas* and *Cyanothece*  
20 species. Soil was also collected from a plot of the Broadbalk Winter Wheat  
21 Experiment (Figure 3D). Shotgun metagenome analysis of DNA extracted from this  
22 soil similarly showed the  $\beta$ PPHy gene sequences to be homologous to those in  
23 *Bacillus*, *Paenibacillus*, *Alteromonas* and *Cyanothece* (Neal & Glendining, 2019).  
24 All the soil plant growth matrix types degraded phytate when added to liquid

1 medium, generating distinct phytate degradation profiles and, but for one,  
2 concomitant accumulation of inorganic phosphate (Figure 3).

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9 Figure 3.

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13 While degradation of phytate by some matrices, Levington's compost (Figure 3A),  
14 Church Farm (Figure 3B) and Bare Fallow (Figure 3D) proceeded to completion or  
15 close to it, indicated by predominant accumulations of Pi, other soils, which  
16 presumably had less abundant or active cohorts of microbes, yielded diagnostic  $\text{InsP}_5$   
17 peaks in the timescale of the experiment. Of the Rothamsted soils, the Broadbalk  
18 soil removed phytate from liquid media such that neither phytate nor lower inositol  
19 phosphates were recovered, at day 0 (not shown). We attribute this to sorption of  
20 phytate to soil particles as this has up to 35% clay content. Nevertheless, by  
21 supplementing the soil/liquid mixture with 1 mM phytate we were subsequently  
22 able to show that the soil and associated microorganisms were capable of processing  
23 added phytate over 8 d (Figure 3E). For this soil, Pi did not accumulate in the  
24 medium - suggesting that the microflora were efficiently scavenging the released  
25 phosphate.

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48 Classification of aggregate phytase activities of soil microbe populations

49 For phytases, the site of initial attack (Figure S1) represents one ontology of enzyme  
50 classification. Enzyme Commission (EC) 3.1.3.26 - 4-phytase, defines enzymes that  
51 remove phosphate from the 1D-4 position of *myo*-inositol hexakisphosphate  
52 (phytate) substrate (the original classification of this enzyme recognizes the detailed  
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1 analysis of enantiomerism of phytate degradation products by cereal activities  
2 (Supplementary information) but the 3.1.3.26 signifier is commonly conflated with  
3 1D-6 phytase (1L-4 phytase) activity. EC 3.1.3.8 - 3-phytase, defines enzymes that  
4 remove phosphate from the 1D-3 position. This distinction makes no consideration  
5 of structural fold or reaction mechanisms.  
6 While bacterial and fungal [1D-] 3-phytases include histidine (acid) phosphatases  
7 with alpha-beta and alpha domain structure, Rossmann fold and characteristic  
8 reaction mechanism involving an attacking histidine nucleophile (His) and proton  
9 donating acidic amino acid (Oh *et al.*, 2004; Mullaney & Ullah, 2007), some [1D-] 3-  
10 phytases possess different structural folds. The  $\beta$ PPHy, exemplified by the enzyme  
11 from *B. amyloliquefaciens*, is a calcium-dependent metalloprotein with catalytic and  
12 structurally important bound  $\text{Ca}^{2+}$  ions (Shin *et al.*, 2001). Equally, histidine  
13 phosphatases include enzymes (phytases) that attack the 1D-6 position, exemplified  
14 by AppA from *E. coli* (Greiner *et al.*, 1993), *Buttiauxella* sp. (Cervin *et al.*, 2008),  
15 *Citrobacter* sp. (Kim *et al.*, 2003; Pontoppidan *et al.*, 2012) and *Hafnia alvei* (Ariza *et*  
16 *al.*, 2013). These are classified as EC 3.1.3.2 acid phosphatases. A comprehensive  
17 review of histidine phosphatases (Rigden, 2008) places fungal phytases and bacterial  
18 acid phosphatases in a branch of a superfamily of functionally diverse histidine  
19 phosphatases which include the enzymes phosphoglycerate mutase and fructose-  
20 2,6-bisphosphatase.  
21 Because simple HPLC resolves the two *meso*-compounds,  $\text{InsP}_5$  [2-OH] and  $\text{InsP}_5$  [5-  
22 OH] from the enantiomeric pairs  $\text{InsP}_5$  [1/3-OH] and  $\text{InsP}_5$  [4/6-OH] (Figure 2C) it is  
23 easy to distinguish between 4-phytases (EC 3.1.3.26) and 3-phytases (EC 3.1.3.8) or  
24 acid phosphatases (EC 3.1.3.2). For example, comparison of Highfield arable soil

1 (Figure 3C) - which yielded predominantly InsP<sub>5</sub> [4/6-OH] - with soil from a plot from  
2 Broadbalk (Figure 3E) - which yielded InsP<sub>5</sub> [1/3-OH] - indicates that the dominant  
3 contributors to phytate degradation in our assays are different enzymes. They  
4 probably represent 6-dephosphorylating histidine (acid) phosphatase (phytase) of EC  
5 3.1.3.2 acid phosphatase (Highfield) and 3-dephosphorylating βPPhy classes  
6 (Broadbalk) (Neal and Glendining, 2019). The absence of InsP<sub>5</sub> [5-OH], diagnostic for  
7 EC 3.1.3.72 - 5-phytase and exemplified by lily pollen alkaline phosphatase  
8 (Barrientos *et al.*, 1994), a eukaryotic MINPP (Mehta *et al.*, 2006), *Bifidobacterium*  
9 *pseudocatenulatum* MINPP (Haros *et al.*, 1999) and *Bacteroides thetaiotaomicron*  
10 MINPP (Stentz *et al.*, 2014), precludes dominant contribution from these classes of  
11 enzyme. Of course, while generation of a peak of e.g., InsP<sub>5</sub> [4/6-OH] could arise  
12 from attack at the [1D-] 6-position by an *E. coli*-like histidine (acid) phosphatase or  
13 from attack at the [1D-] 4-position by an enzyme with similar activity to the cereal  
14 phytase, the inclusion of cycloheximide in our media prevents eukaryotic growth.  
15 The situation is further compounded by the first report of a bacterial PAPhy with  
16 predominant InsP<sub>5</sub> [4/6-OH] product, harboured by a soil earthworm castmicrobe  
17 with similarity to *Sphingobium yanoikuyae* (Nasrabadi *et al.*, 2018).

18

19 Identification of *Acinetobacter* and *Buttiauxella* sp. in soil samples

20 In an extension to the above analyses, we amplified and sequenced 16S rRNA genes  
21 from isolates of agricultural and Church Farm soils giving HPLC profiles of Figures 2  
22 and 3B. These isolates designated AC1-2 and CH-10-6-4 (both from 10<sup>6</sup> dilutions of  
23 these soil samples) are both *Gammaproteobacteria*. The amplified 16S rRNA gene of  
24 strain AC1-2 (MT450216) was identical to that of *Acinetobacter* sp. strain YAZ49,

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1  
2 1 *Acinetobacter calcoaceticus* strain EB11, *Acinetobacter calcoaceticus* strain P19 and  
3  
4 2 that recovered from whole genome sequencing (JABFFO000000000) of the parent  
5  
6 3 isolate AC1-2. The 16S rRNA gene of strain CH-10-6-4 was identical to that in  
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8 4 *Buttiauxella agrestis* strain EB112, *Buttiauxella* sp. CL\_136\_AN\_40 and *Buttiauxella*  
9  
10 5 sp. SA\_136\_AN\_45 (MT450213-MT450215). To investigate the distribution of  
11  
12 6 different phytases in these two genera, BLAST searches were conducted using  
13  
14 7 ratified examples of each of five phytase families, restricting searches only to  
15  
16 8 *Acinetobacter* and *Buttiauxella* species (Table 1). For PAPHy, we followed Nasrabadi  
17  
18 9 et al. (2018) using *Lupinus luteus* (AJ505579) as query to blast *Sphingobium* spp.  
19  
20 10 genomes, returning hits with percentage identity 23–26% with E-value of  $5^{-12}$  to  $9^{-10}$ .  
21  
22 11 Subsequently, the full gene from *Sphingobium yanoikuyae* (CP060122) was used as  
23  
24 12 query of *Acinetobacter* and *Buttiauxella* spp. These searches indicated a divergent  
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26 13 distribution of phytase families between the two organisms. *Acinetobacter* species  
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28 14 were predominantly associated with MINPP and  $\beta$ PPhy, while histidine (acid)  
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30 15 phosphatase was the only phytase family associated with *Buttiauxella* species.  
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41 17 Table 1. Frequency of canonical phytase classes between referenced genomes  
42  
43 18 (Protein Blast of Non-Redundant Protein Sequences at NCBI) of *Acinetobacter* and  
44  
45 19 *Buttiauxella* spp.

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48 20 The reference sequences used were: Multiple Inositol Polyphosphate Phosphatase  
49  
50 21 (MINPP), *Bacteroides thetaiotaomicron* (WP\_009040027); Histidine (Acid)  
51  
52 22 Phosphatase (HAP), *Citrobacter amalonaticus* (DQ975370.1;  $\beta$ -propeller phytase  
53  
54 23 ( $\beta$ PPhy), *Bacillus amyloliquefaciens* (WP\_013352583); Protein Tyrosine Phosphatase  
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1 (PTP), *Selenomonas lactificex* (ABC69367) and Purple Acid Phytase (PAPhy),  
 2 *Sphingobium yanoikuyae* (CP060122).

		<i>Acinetobacter</i> spp.	<i>Buttiauxella</i> spp.
3			
4	MINPP	445	0
5	HAP	9	24
6	$\beta$ PPhy	80	0
7	PTP	0	0
8	PAPhy	1	0

9  
 10 To interrogate further the phylogenetic separation of histidine (acid) phosphatase  
 11 between *Acinetobacter* spp. and *Buttiauxella* spp., revealed in Table 1, a diverse  
 12 selection of accessions (reference genomes) of each sp. were searched by tblastn in  
 13 NCBI with the different phytase reference sequences of Table 1 as query. The results  
 14 are shown in Table S1, in which crosses indicate the presence of the different  
 15 phytase proteins in selected genome-sequenced *Acinetobacter* and *Buttiauxella*  
 16 strains yielding E value < 0.00005. Only a single histidine (acid) phosphatase was  
 17 present in the *Buttiauxella* genomes analysed. These were either AppA phytases or  
 18 bifunctional glucose-1-phosphatase/inositol phosphatases. The phytase  
 19 complements of *Acinetobacter* genomes were more varied, revealing the presence  
 20 of all different classes of phytase with the exception of Protein Tyrosine  
 21 Phosphatase. Additionally, while predominantly only containing a single phytase,  
 22 there were some cases of *Acinetobacter* sp. containing two different classes of  
 23 phytase: either MINPP and  $\beta$ PPhy, or histidine (acid) phosphatase and  $\beta$ PPhy.

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2 1 Phytate degradation profiles of isolated *Acinetobacter* and *Buttiauxella* strains reveal  
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4 2 distinct histidine phosphatase activities  
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7 3 To confirm the ability of identified isolates bearing defined cohort(s) of phytase(s) to  
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9 4 degrade phytate and to characterize those enzyme activities, the isolates  
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12 5 *Acinetobacter* sp. AC1-2 (AC1-2) and *Buttiauxella* sp. isolate CH-10-6-4 were  
13  
14 6 incubated with phytate and subjected to HPLC analysis (Figure 4A,B). This  
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16  
17 7 demonstrated that enzymes associated with AC1-2 are promiscuous in their site of  
18  
19 8 initial attack on phytate substrate, yielding among InsP<sub>5</sub> isomers a dominant 4/6-OH  
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22 9 peak, a smaller 5-OH peak and little to no detectable degradation at the 1/3-position  
23  
24 10 (Figure 4A). Interestingly, strain CH-10-6-4 did not show any phytase activity in  
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26  
27 11 minimal medium, it did however degrade 1 mM phytate when incubated in a 20 mM  
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29 12 Tris-HCl and 0.1% NaCl solution (Figure 4B).

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34 14 Figure 4.

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39 16 The *Buttiauxella* strain CH-10-6-4 (Figure 4B) showed a high specificity towards the  
40  
41 17 initial position of attack on phytate, generating InsP<sub>5</sub> [4/6-OH] predominantly among  
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43  
44 18 InsP<sub>5</sub> products, consistent with the published properties of *Buttiauxella* phytase  
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46 19 (Cervin *et al.*, 2008) and its industrial use (Ushasree *et al.*, 2017; Herrmann *et al.*,  
47  
48 20 2019).

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51 21 While both the *Acinetobacter* and *Buttiauxella* strains showed preferential 1D-4/6  
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53 22 selectivity of attack on phytate, they differ in terms of the resulting InsP<sub>4</sub>  
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56 23 intermediates: the *Acinetobacter* strain produced four InsP<sub>4</sub> intermediates, whilst  
57  
58 24 the *Buttiauxella* strain produced two, a predominant peak with the chromatographic  
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1 properties of D/L-Ins(2,3,4,5)P<sub>4</sub> and a minor peak with that of D/L-Ins(1,2,3,4)P<sub>4</sub>.  
2 Again, HPLC can be shown to distinguish between classes of phytase without  
3 assistance of 16S rRNA gene. The phytate degradation profile of the *Buttiauxella*  
4 isolate is characteristic of 1D-6-directed histidine (acid) phosphatase, that of the  
5 *Acinetobacter* strain was indicative of the MINPP subclass of the histidine (acid)  
6 phosphatases (Tayamo-Ramos *et al.*, 2012; Stentz *et al.*, 2014). Congruent with these  
7 predictions, strain CH-10-6-4 was shown by PCR to contain an histidine (acid)  
8 phosphatase, 100% identical at the amino acid level to that in *Buttiauxella*  
9 *ferragutiae*. Furthermore, the genome sequence of AC1-2 was shown to encode a  
10 MINPP 98.28% identical at amino acid level to that in *Acinetobacter calcoaceticus*.  
11  
12 With this additional information we undertook an alignment of phytase protein  
13 sequences for thirty-one histidine (acid) phosphatases and twenty-seven MINPPs  
14 using the online multisequence alignment tool MAFFT (Kato *et al.*, 2019), reporting  
15 the output as an Interactive Tree of Life, iTOL (Letunic and Bork, 2019) (Figure 5).  
16 The results of this analysis split MINPP sequences into two clades, those whose  
17 origins are from animals and plants (Cho *et al.*, 2006; Dionisio *et al.*, 2007), and those  
18 from bacteria (Haros *et al.*, 2009; Tayamo-Ramos *et al.*, 2012; Stentz *et al.*, 2014).  
19 Both are distinct from bacterial histidine (acid) phosphatases, with bacterial MINPPs  
20 more closely related to eukaryotic MINPPs than bacterial histidine (acid)  
21 phosphatases. Of the bacterial MINPPs, the *Acinetobacter* enzyme was more deeply  
22 rooted than the MINPPs of previously characterized gut commensals *Bifidobacter*  
23 and *Bacteroides* spp.

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2 1 Figure 5.  
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7 3 Improved, predictive HPLC-based screening for phytases  
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9 4 The foregoing analyses highlight considerations that apply to culture-dependent  
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11 5 isolation of phytases, here from environmental samples. The methods described  
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13 6 overcome problems associated with the purity of phytate substrate (Madsen *et al.*,  
14  
15 7 2019) and 'zone-clearing' assays (Fredrikson *et al.*, 2002). Nevertheless, PSM can be  
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17 8 a useful media for obtaining a diverse set of bacteria (Greiner *et al.*, 1997;  
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19 9 Richardson & Hadobas, 1997; Kerovuo *et al.*, 1998) or for the screening of  
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21 10 engineered bacteria and plants (Shulse *et al.*, 2019).  
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24 11 Here, the opportunity to characterize enzyme activity of isolates before functional  
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26 12 cloning, expression, purification, subsequent verification of catalytic activity, is a  
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28 13 considerable shortcut that focuses attention among isolates on those with *bona fide*  
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30 14 phytase activity. Moreover, sequencing of the *Acinetobacter* and *Buttiauxella* strains  
31  
32 15 revealed the power of this HPLC-based screening strategy to illuminate phytase  
33  
34 16 diversity. The two different histidine phosphatases, MINPP and histidine (acid)  
35  
36 17 phosphatase, are typical of the families of enzymes identified in sequenced genera.  
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38 18 The assembled sequenced genome (JABFFO000000000) of the *Acinetobacter* strain  
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40 19 AC1-2 harbours a single histidine (acid) phosphatase of the MINPP class, rather than  
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42 20 a canonical histidine (acid) phosphatase.  
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44 21 The enzyme bears a hepta-peptide catalytic site sequence motif of RHGSRGL: RHG is  
45  
46 22 characteristic of the histidine phosphatase superfamily (Rigden, 2008), and the  
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48 23 proton donor motif is HAE, with glutamate replacing aspartate of the HD motif of  
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50 24 histidine (acid) phosphatases. AC1-2 MINPP is more closely related to eukaryotic,  
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1 plant and animal MINPP than it is to bacterial histidine (acid) phosphatases.  
2 Significantly, the only prior functional identification of a bacterial MINPP is that of  
3 the human gut commensals *Bifidobacterium pseudocatenulatum* and *longum* subsp.  
4 *infantis* (Haros *et al.*, 2009; Tamayo-Ramos *et al.*, 2012) and *Bacteroides*  
5 *thetaiotaomicron* (Stentz *et al.*, 2014) that share the HAE motif. Other homologues  
6 can be found among the *Actinobacteria*, *Betaproteobacteria* and  
7 *Gammaproteobacteria* (Tamayo-Ramos *et al.*, 2012; Stentz *et al.*, 2014). Our  
8 identification of significant contribution of MINPP to aggregate environmental  
9 phytase activity and to *Acinetobacter*, particularly, serves to highlight novel  
10 biotechnological opportunity of exploitation of environmental samples.  
11 *Acinetobacter* spp. are commonly cited in context, but in no means as the principal  
12 agent, of enhanced biological phosphorus removal (Seviour *et al.*, 2003). They  
13 harbour a polyphosphate kinase *ppk* that is induced by Pi starvation (Trelstad *et al.*  
14 1999). It seems likely therefore that the function of MINPP may be related to Poly P  
15 accumulation in soil *Acinetobacter*.  
16 The second isolate was identified as a *Buttiauxella* strain and comparison with  
17 published genomes of similar strains revealed, in contrast, a single canonical  
18 histidine (acid) phosphatase. BLAST searches of *Buttiauxella* accessions for all  
19 phytase classes yielded only histidine (acid) phosphatase with E values less than  $10^{-68}$ .  
20 These were of the *E. coli* AppA family histidine acid phosphatase (Lim *et al.*, 2000)  
21 with RHGVRAP and HDTN motifs, or bifunctional glucose 1-phosphatase/phytase  
22 (Golovan *et al.*, 2000; Lee *et al.*, 2003) class with RHNLRAP (similar to RANLRAP (Lee  
23 *et al.*, 2003)) and HDSN (similar to HDQN (Lee *et al.*, 2003)) motifs. The *Buttiauxella*  
24 sp. AppA and its engineered variants (Cervin *et al.*, 2008) are already a commercial

1 product used widely to improve pig and poultry performance (e.g. Adedokun *et al.*,  
2 2015). Other bacterial AppA enzymes, e.g., from *E. coli* and *Citrobacter* spp., are used  
3 similarly (Sommerfeld *et al.*, 2018; da Silva *et al.*, 2019). Our unbiased, for phytase  
4 class, screening approach is clearly capable of identifying candidate phytases with  
5 potential as commercial leads.

## 6 7 Experimental Procedures

### 8 Media

9 Agar was obtained from Sigma (UK). Tryptone and yeast extract for preparation of  
10 Lysogeny broth were obtained from Formedium (UK).

### 11 12 Preparations of Soil Cultures

13 Soil (0.5g) was added to 10 mL of minimal media, pH 7, in a 30 mL universal. The  
14 base media, modified from (Neal *et al.*, 2017), comprised: 18.7 mM NH<sub>4</sub>Cl, 8.6 mM  
15 NaCl, 1 mM MgSO<sub>4</sub>, 0.1 mM CaCl<sub>2</sub>, 1 mM succinate, 1mM glucose, 1mM sucrose,  
16 1mM pyruvate, pH 7 and 1mM InsP<sub>6</sub>. The media was supplemented with vitamins:  
17 10 µL of vitamin solution (containing 10 mg pyridoxine.HCl, 5 mg thiamine.HCl, 5 mg  
18 riboflavin, 5 mg para-amino benzoic acid, 5 mg nicotinic acid, 2 mg vitamin B12, 2 mg  
19 folic acid, L<sup>-1</sup>) and with micronutrients: 10 µL (2 g nitriloacetic acid, 1 g MnSO<sub>4</sub>.6H<sub>2</sub>O,  
20 0.8 g Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub>, 0.2 g CoCl<sub>2</sub>.6H<sub>2</sub>O, 0.2 g ZnSO<sub>4</sub>.7H<sub>2</sub>O, 20 mg CuCl<sub>2</sub>.2H<sub>2</sub>O, 20mg  
21 NiCl<sub>2</sub>.6H<sub>2</sub>O, Na<sub>2</sub>MoO<sub>4</sub>.2H<sub>2</sub>O L<sup>-1</sup>). The medium included 0.1-0.2 mg mL<sup>-1</sup> cycloheximide  
22 to inhibit fungal growth. Soil suspensions were incubated under shaking at 180 RPM  
23 and 30 °C for six days, taking samples each day. Samples were diluted and plated  
24 onto LB media and incubated for 2 days at 30 °C.

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## 2 Acid-extraction of phytate from Phytase Specific Media Plates

3 Bacterial cells were washed off the plate using dH<sub>2</sub>O and 100 mg samples of agar  
4 were extracted with 400 µL 0.8 M HCl with vortexing after disruption of the agar  
5 with a plastic stirrer. Samples were extracted for 15 min at room temperature and  
6 centrifuged at 13,000 x *g* for one minute. The supernatant was removed with a HPLC  
7 needle and syringe and filtered through a 13mm diameter 0.45 µm pore PTFE  
8 syringe filter (Kinesis, UK) into a borosilicate glass HPLC vial (Chromacol03-FISV(A)).

9

## 10 Preparation of Soil Cultures for HPLC Analysis

11 Five hundred µL of a well-mixed soil culture in media was centrifuged at 13000 x *g*  
12 for 5 minutes. The supernatant was filtered through a 13mm diameter 0.45 µm pore  
13 PTFE syringe filter (Kinesis, UK), centrifuged again and an aliquot (200 µL) dispensed  
14 into an HPLC vial.

15

## 16 HPLC Analysis of Inositol Phosphates

17 Inositol phosphates were analysed according to (Whitfield *et al.*, 2018).

18 Chromatography data was exported as *x,y* data and redrawn in GraphPad Prism

19 v.6.0.

20

## 21 16S amplification

22 Single bacterial colonies were purified, and their 16S rRNA gene amplified using the

23 primers 28F (5'-GAGTTTGATCNTGGCTCAG-3') and 519R (5'-

24 GWNTTACNGCGGCKGCTG-3') from genomic DNA using colony PCR. The PCR

1 generated a single band resolved on a 1 % agarose gel and this was purified using a  
2 QIAquick Gel Extraction kit (QIAGEN). Sequencing of these PCR products at Eurofins  
3 (MWG, Germany) identified the two isolates further examined in this study as strains  
4 of *Acinetobacter* sp. and *Buttiauxella* sp. To confirm that the isolated *Buttiauxella* sp  
5 CH-10-6-4 contained a histidine (acid) phosphatase, primers were designed to the  
6 *appA* gene using sequenced *Buttiauxella* spp. genomes (*Buttiauxella* sp. JUb87,  
7 *Buttiauxella* sp. A111, *Buttiauxella agrestis*, *Buttiauxella ferragutiae*, *Buttiauxella*  
8 *brennerae*, *Buttiauxella gaviniae*, *Buttiauxella noackiae*, *Buttiauxella* sp. BIGb0552,  
9 *Buttiauxella* sp. 3AFRM03). These primers (Forward 5'-GCG AGA ART TTC AAC ARC  
10 AGG -3', Reverse 5'-GTG YCC GGC AAK AAA CAG G-3') were used to amplify a 725 bp  
11 product from the *Buttiauxella* sp. isolate. These PCR products were sequenced by  
12 Eurofins and their identity to ratified *Buttiauxella* spp. *appA* genes was established  
13 by BLAST analysis. The sequence was deposited in GenBank under the accession  
14 MT680195.

## 15 Sequencing of Strains

16 The *Acinetobacter* sp. strain AC1-2 genome was sequenced by MicrobesNG  
17 (University of Birmingham, UK) using Illumina technology. This Whole Genome  
18 Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession  
19 JABFFO000000000. The version described in this paper is version JABFFO010000000.  
20 Genomic completeness was analysed using BUSCO v3 (Simao *et al.*, 2015), an open-  
21 source software that provides quantitative measures for genomic completeness  
22 based on evolutionarily informed expectations of gene content from near-universal  
23 single-copy orthologs selected. The *Acinetobacter* sp. strain AC1-2 completeness was  
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2 1 measured at 98, and 98.9% from both BUSCO's bacterial and *Gammaproteobacteria*  
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4 2 databases, respectively.  
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32 13 Author Contributions  
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34 14 GDR performed experiments, curated data and provided an original draft. JDT  
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36 15 supervised experiments and edited the manuscript. ALN supervised experiments,  
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38 16 curated data and wrote the manuscript. CAB secured funding, supervised  
39  
40 17 experiments, and wrote the manuscript.  
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## References

- Adedokun, S.A., Owusu-Asiedu, A., Ragland, D., Plumstead, P., Adeola, O. (2015) The efficacy of a new 6-phytase obtained from *Buttiauxella* spp. expressed in *Trichoderma reesei* on digestibility of amino acids, energy, and nutrients in pigs fed a diet based on corn, soybean meal, wheat middlings, and corn distillers' dried grains with solubles. *J Anim Sci* **93**(1): 168-75.
- Ariza, A., Moroz, O.V., Blagova, E.V., Turkenburg, J.P., Waterman, J., Roberts, S.M., Vind, J. *et al.* (2013) Degradation of phytate by the 6-phytase from *Hafnia alvei*: A combined structural and solution study. *PLoS ONE* **8**(5): e65062. doi: 10.1371/journal.pone.0065062.
- Bae, H.D., Yanke, L.J., Cheng, K.J., Selinger, L.B. (1999) A novel staining method for detecting phytase activity. *Journal of Microbiological Methods* **39**: 17-22.
- Barrientos, L., Scott, J.J., Murthy, P.P. 1994. Specificity of hydrolysis of phytic acid by alkaline phytase from lily pollen. *Plant Physiol* **106**(4): 1489-95.
- Castillo Villamizar, G.A., Funkner, K., Nacke, H., Foerster, K., Daniel, R. (2019a) Functional metagenomics reveals a new catalytic domain, the metallo-beta-lactamase superfamily domain, associated with phytase activity. *mSphere* **4**: 10.1128/mSphere.00167-19
- Castillo Villamizar, G.A., Nacke, H., Griese, L., Taberner, L., Funkner, K., Daniel, R. (2019b) Characteristics of the first protein tyrosine phosphatase with phytase activity from a soil metagenome. *Genes (Basel)* **10**: 10.3390/genes10020101
- Cervin, M.A., Kensch, O., Kettling, U., Leuthner, B., Miasnikov, A., Pellengahr, K. (2008) Variant *Buttiauxella* sp. phytases having altered properties, Danisco US INC Genencor DIV <https://lens.org/056-995-949-255-887>



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55  
56  
57  
58  
59  
60

- 1 Chen, X., Jiang, N., Condrón, L.M., Dunfield, K.E., Chen, Z., Wang, J., Chen, L. (2019)  
2 Impact of long-term phosphorus fertilizer inputs on bacterial phoD gene  
3 community in a maize field, Northeast China. *Sci Total Environ* **669**: 1011-  
4 1018.
- 5 Cho, J., Choi, K., Darden, T., Reynolds, P.R., Petite, J.N., Shears, S.B. (2006) Avian  
6 multiple inositol polyphosphate phosphatase is an active phytase that can be  
7 engineered to help ameliorate the planet's "phosphate crisis". *J Biotechnol*  
8 **126**: 248-259.
- 9 Cosgrove, D.J. (1980) Inositol phosphates, Their Chemistry, Biochemistry and  
10 Physiology. Elsevier, Amsterdam.
- 11 Cosgrove, D.J., Irving, G.C.J., Bromfield, S.M. (1970) Inositol phosphate phosphatases  
12 of microbiological origin. The isolation of soil bacteria having inositol  
13 phosphate phosphatase activity. *Australian Journal of Biological Sciences* **23**:  
14 339-344.
- 15 da Silva, C.A., Callegari, M.A., Dias, C.P., Bridi, A.M., Pierozan, C.R., Foppa, L. *et al.*  
16 (2019) Increasing doses of phytase from *Citrobacter braakii* in diets with  
17 reduced inorganic phosphorus and calcium improve growth performance and  
18 lean meat of growing and finishing pigs. *PLoS One* **14**(5): e0217490.
- 19 Dionisio, G., Holm, P.B., Brinch-Pedersen, H. (2007) Wheat (*Triticum aestivum* L.) and  
20 barley (*Hordeum vulgare* L.) multiple inositol polyphosphate phosphatases  
21 (MINPPs) are phytases expressed during grain filling and germination. *Plant*  
22 *Biotechnology Journal* **5**: 325-338.

- 1  
2 1 Dorsch, J.A., Cook, A., Young, K.A., Anderson, J.M., Bauman, A.T., Volkmann, C.J. *et*  
3  
4 2 *al.* (2003) Seed phosphorus and inositol phosphate phenotype of barley low  
5  
6 3 phytic acid genotypes. *Phytochemistry* **62**: 691-706.  
7  
8  
9 4 Fredrikson, M., Andlid, T., Haikara, A., Sandberg, A.S. (2002) Phytate degradation by  
10  
11 5 micro-organisms in synthetic media and pea flour. *Journal of Applied*  
12  
13 6 *Microbiology* **93**: 197-204.  
14  
15  
16 7 Golovan, S., Wang, G., Zhang, J., Forsberg, C.W. (2000) Characterization and  
17  
18 8 overproduction of the *Escherichia coli* appA encoded bifunctional enzyme  
19  
20 9 that exhibits both phytase and acid phosphatase activities. *Can J Microbiol*  
21  
22 10 **46**(1): 59-71.  
23  
24  
25 11 Greiner, R., Haller, E., Konietzny, U., Jany, K.D. (1997) Purification and  
26  
27 12 characterization of a phytase from *Klebsiella terrigena*. *Arch Biochem Biophys*  
28  
29 13 **341**: 201-206.  
30  
31  
32 14 Greiner, R., Konietzny, U., Jany, K.D. (1993) Purification and characterization of two  
33  
34 15 phytases from *Escherichia coli*. *Arch Biochem Biophys* **303**(1): 107-13.  
35  
36  
37 16 Guerrand, D. (2018) Economics of food and feed enzymes: Status and perspectives.  
38  
39 17 Status and perspectives. in: *Enzymes in Human and Animal Nutrition:*  
40  
41 18 *Principles and Perspectives*, Elsevier Inc., pp. 487-514.  
42  
43  
44 19 Haros, M., Carlsson, N-G., Almgren, A., Larsson-Alminger, M., Sandberg, A-S., Andlid,  
45  
46 20 A. (2009) Phytate degradation by human gut isolated *Bifidobacterium*  
47  
48 21 *pseudocatenulatum* ATCC27919 and its probiotic potential. *International*  
49  
50 22 *Journal of Food Microbiology* **135**: 7-14.  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

- 1  
2 1 Herrmann, K.R., Ruff, A.J., Infanzón, B., Schwaneberg, U. (2019) Engineered phytases  
3  
4 2 for emerging biotechnological applications beyond animal feeding. in:  
5  
6 3 *Applied Microbiology and Biotechnology* **103**: 6435-6448.  
7  
8  
9 4 Howson, S.J., Davis, R.P. (1983) Production of phytate-hydrolysing enzyme by some  
10  
11 5 fungi. *Enzyme and Microbial Technology* **5**: 377-382.  
12  
13 6 Huang, H., Luo, H., Yang, P., Meng, K., Wang, Y., Yuan, T. *et al.* (2006) A novel  
14  
15 7 phytase with preferable characteristics from *Yersinia intermedia*. *Biochem*  
16  
17 8 *Biophys Res Commun* **350**(4): 884-9.  
18  
19  
20 9 Irving, G.C., Cosgrove, D.J. (1972) Inositol phosphate phosphatases of microbiological  
21  
22 10 origin: the inositol pentaphosphate products of *Aspergillus ficuum* phytases. *J*  
23  
24 11 *Bacteriol* **112**(1): 434-8.  
25  
26  
27 12 Iyer, B., Rajput, M.S., Rajkumar, S. (2017) Effect of succinate on phosphate  
28  
29 13 solubilization in nitrogen fixing bacteria harbouring chick pea and their effect  
30  
31 14 on plant growth. *Microbiological Research* **202**: 43-50.  
32  
33  
34 15 Katoh, K., Rozewicki, J., Yamada, K.D. (2019) MAFFT online service: multiple  
35  
36 16 sequence alignment, interactive sequence choice and visualization. *Brief*  
37  
38 17 *Bioinform* **20**(4): 1160-1166.  
39  
40  
41 18 Kerovuo, J., Lauraeus, M., Nurminen, P., Kalkkinen, N., Apajalahti, J. (1998) Isolation,  
42  
43 19 characterization, molecular gene cloning, and sequencing of a novel phytase  
44  
45 20 from *Bacillus subtilis*. *Applied and Environmental Microbiology* **64**: 2079-  
46  
47 21 2085.  
48  
49  
50 22 Kim, H.W., Kim, Y.O., Lee, J.H., Kim, K.K., Kim, Y.J. (2003) Isolation and  
51  
52 23 characterization of a phytase with improved properties from *Citrobacter*  
53  
54 24 *braakii*. *Biotechnol Lett* **25**(15): 1231-4.  
55  
56  
57  
58  
59  
60

- 1  
2 1 Konietzny, U; Greiner, R. (2002) Molecular and catalytic properties of phytate-  
3  
4 2 degrading enzymes (phytases) *Int J Food Sci Tech* **37**: 791-812.  
5  
6  
7 3 Kumar, V., Singh, P., Jorquera, M.A., Sangwan, P., Kumar, P., Verma, A.K., Agrawal, S.  
8  
9 4 (2013) Isolation of phytase-producing bacteria from Himalayan soils and their  
10  
11 5 effect on growth and phosphorus uptake of Indian mustard (*Brassica juncea*).  
12  
13 6 *World Journal of Microbiology and Biotechnology* **29**: 1361-1369.  
14  
15  
16  
17 7 Lee, D.C., Cottrill, M.A., Forsberg, C.W., Jia, Z. (2003) Functional insights revealed by  
18  
19 8 the crystal structures of *Escherichia coli* glucose-1-phosphatase. *J Biol Chem*  
20  
21 9 **278**(33): 31412-8.  
22  
23  
24 10 Lehmann, M., Kostrewa, D., Wyss, M., Brugger, R., D'Arcy, A., Pasamontes, L., van  
25  
26 11 Loon, A.P. (2000) From DNA sequence to improved functionality: using  
27  
28 12 protein sequence comparisons to rapidly design a thermostable consensus  
29  
30 13 phytase. *Protein Eng* **13**(1): 49-57.  
31  
32  
33  
34 14 Lei, X.G., Porres, J.M. (2003) Phytase enzymology, applications, and biotechnology.  
35  
36 15 *Biotechnology Letters* **25**: 1787-1794.  
37  
38  
39 16 Letunic, I., Bork, P. (2019) Interactive Tree of Life (iTOL) v4: recent updates and new  
40  
41 17 developments *Nucleic Acids Research* **47** (W1): 256-259. 10.1093/nar/gkz239.  
42  
43  
44 18 Lim, D., Golovan, S., Forsberg, C.W., Jia, Z. (2000) Crystal structures of *Escherichia*  
45  
46 19 *coli* phytase and its complex with phytate. *Nat Struct Biol* **7**(2): 108-13.  
47  
48  
49 20 Madsen, C.K., Brearley, C.A., Brinch-Pedersen, H. (2019) Lab-scale preparation and  
50  
51 21 QC of phytase assay substrate from rice bran. *Analytical Biochemistry* **578**: 7-  
52  
53 22 12.  
54  
55  
56  
57  
58  
59  
60

- 1  
2 1 Mehta, B.D., Jog, S.P., Johnson, S.C., Murthy, P.P. (2006) Lily pollen alkaline phytase  
3  
4 2 is a histidine phosphatase similar to mammalian multiple inositol  
5  
6 3 polyphosphate phosphatase (MINPP). *Phytochemistry* **67**: 1874-1886.  
7  
8  
9 4 Menezes-Blackburn, D., Giles, C., Darch, T., George, T.S., Blackwell, M., Stutter, M. *et*  
10  
11 5 *al.* 2018. Opportunities for mobilizing recalcitrant phosphorus from  
12  
13 6 agricultural soils: a review. *Plant and Soil* **427**: 5-16.  
14  
15  
16 7 Mullaney, E.J., Ullah, A.H. (2003) The term phytase comprises several different  
17  
18 8 classes of enzymes. *Biochem Biophys Res Commun* **312**(1): 179-84.  
19  
20  
21 9 Mullaney, E.J., Ullah, A.H.J. (2007) Phytases: attributes, catalytic mechanisms, and  
22  
23 10 applications. *Inositol phosphates: linking agriculture and the environment*.  
24  
25 11 *CAB International, Oxfordshire, United Kingdom*, 97-110.  
26  
27  
28 12 Nagul, E.A., McKelvie, I.D., Worsfold, P., Kolev, S.D. (2015) The molybdenum blue  
29  
30 13 reaction for the determination of orthophosphate revisited: Opening the  
31  
32 14 black box. *Analytica Chimica Acta* **890**: 60-82.  
33  
34  
35 15 Nasrabadi, R.G., Greiner, R., Yamchi, A., Roshan, E.N. (2018) A novel purple acid  
36  
37 16 phytase from an earthworm cast bacterium. *J Sci Food Agric* **98**: 3667-3674.  
38  
39  
40 17 Neal, A.L., Glendining, M.J. (2019) Calcium exerts a strong influence upon  
41  
42 18 phosphohydrolase gene abundance and phylogenetic diversity in soil. *Soil*  
43  
44 19 *Biology and Biochemistry* **139**: 10.1016/j.soilbio.2019.107613  
45  
46  
47 20 Neal, A.L., Rossmann, M., Brearley, C., Akkari, E., Guyomar, C., Clark, I.M. *et al.*  
48  
49 21 (2017) Land-use influences phosphatase gene microdiversity in soils.  
50  
51 22 *Environmental Microbiology* **19**: 2740-2753.  
52  
53  
54  
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57  
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54  
55  
56  
57  
58  
59  
60
- 1 Oh, B.C., Choi, W.C., Park, S., Kim, Y.O., Oh, T.K. (2004) Biochemical properties and  
2 substrate specificities of alkaline and histidine acid phytases. *Applied*  
3 *Microbiology and Biotechnology* **63**(4): 362-372.
- 4 Pandey, A., Szakacs, G., Soccol, C.R., Rodriguez-Leon, J.A., Soccol, V.T. (2001)  
5 Production, purification and properties of microbial phytases. *Bioresource*  
6 *Technology* **77**(3): 203-14.
- 7 Pontoppidan, K., Glitsoe, V., Guggenbuhl, P., Quintana, A.P., Nunes, C.S., Pettersson,  
8 D., Sandberg, A.S. (2012) In vitro and in vivo degradation of *myo*-inositol  
9 hexakisphosphate by a phytase from *Citrobacter braakii*. *Arch Anim Nutr*  
10 **66**(6): 431-44.
- 11 Powar, V.K., Jagannathan, V. (1982) Purification and properties of phytate-specific  
12 phosphatase from *Bacillus subtilis*. *Journal of Bacteriology* **151**: 1102-1108.
- 13 Puppala, K.R., Bhavsar, K., Sonalkar, V., Khire, J.M., Dharme, M.S. (2019)  
14 Characterization of novel acidic and thermostable phytase secreting  
15 *Streptomyces* sp. (NCIM 5533) for plant growth promoting characteristics.  
16 *Biocatalysis and Agricultural Biotechnology* **18**: 101020.
- 17 Raboy, V., Dickinson, D.B. (1993) Phytic acid levels in seeds of *Glycine max* and *G. soja*  
18 as influenced by phosphorus status. *Crop Science* **33**: 1300-1305.
- 19 Ragot, S.A., Kertesz, M.A., Bunemann, E.K. (2015) phoD alkaline phosphatase gene  
20 diversity in soil. *Applied and Environmental Microbiology* **81**: 7281-7289.
- 21 Ranjard, L., Poly, F., Nazaret, S. (2000) Monitoring complex bacterial communities  
22 using culture-independent molecular techniques: Application to soil  
23 environment. *Research in Microbiology* **151**(3): 166-177.

- 1  
2 1 Richardson, A.E., Hadobas, P.A. (1997) Soil isolates of *Pseudomonas* spp. that utilize  
3  
4 2 inositol phosphates. *Can J Microbiol* **43**(6): 509-16.  
5  
6  
7 3 Rigden, D.J. (2008) The histidine phosphatase superfamily: structure and function.  
8  
9 4 *Biochem J* **409**(2): 333-48.  
10  
11  
12 5 Sarikhani, M.R., Malboobi, M.A., Aliasgharzag, N., Greiner, R. 2019. Identification of  
13  
14 6 two novel bacterial phosphatase-encoding genes in *Pseudomonas putida*  
15  
16 7 strain P13. *J Appl Microbiol* **127**: 1113-1124.  
17  
18  
19 8 Seviour, R.J., Mino, T., Onuki, M. (2003) The microbiology of biological phosphorus  
20  
21 9 removal in activated sludge systems. *FEMS Microbiology Reviews* **27**: 99-127.  
22  
23  
24 10 Shin, S., Ha, N.C., Oh, B.C., Oh, T.K., Oh, B.H. (2001) Enzyme mechanism and catalytic  
25  
26 11 property of beta propeller phytase. *Structure* **9**: 851-8.  
27  
28  
29 12 Shulse, C.N., Chovatia, M., Agosto, C., Wang, G., Hamilton, M., Deutsch, S. *et al.*  
30  
31 13 (2019) Engineered root bacteria release plant-available phosphate from  
32  
33 14 phytate. *Applied and Environmental Microbiology* **85**(18): doi:  
34  
35 15 10.1128/AEM.01210-19  
36  
37  
38  
39 16 Simao, F.A., Waterhouse, R.M., Ioannidis, P., Kriventseva, E.V., Zdobnov, E.M. (2015)  
40  
41 17 BUSCO: assessing genome assembly and annotation completeness with  
42  
43 18 single-copy orthologs. *Bioinformatics* **31**(19): 3210-3212.  
44  
45  
46 19 Sommerfeld, V., Kunzel, S., Schollenberger, M., Kuhn, I., Rodehutsord, M. (2018)  
47  
48 20 Influence of phytase or *myo*-inositol supplements on performance and  
49  
50 21 phytate degradation products in the crop, ileum, and blood of broiler  
51  
52 22 chickens. *Poult Sci* **97**: 920-929.  
53  
54  
55  
56 23 Stentz, R., Osborne, S., Horn, N., Li, A.W.H., Hautefort, I., Bongaerts, R. *et al.* (2014) A  
57  
58 24 Bacterial homolog of a eukaryotic inositol phosphate signaling enzyme  
59  
60

- 1  
2 1 mediates cross-kingdom dialog in the mammalian gut. *Cell Reports* **6**: 646-  
3  
4 2 656.  
5  
6  
7 3 Tamayo-Ramos, J.A., Mario Sanz-Penella, J., Yebra, M.J., Monedero, V., Haros, M.  
8  
9 4 (2012) Novel phytases from *Bifidobacterium pseudocatenulatum* ATCC 27919  
10  
11 and *Bifidobacterium longum* subsp. *infantis* ATCC 15697 *Applied and*  
12  
13 *Environmental Microbiology* **78**: 5013-5015.  
14  
15  
16  
17 7 Trelstad, P.L., Purdhani, P., Geissdörfer, W., Hillen, W. and Keasling, J.D. (1999)  
18  
19 8 Polyphosphate kinase of *Acinetobacter* sp. strain ADP1: purification and  
20  
21 9 characterization of the enzyme and its role during changes in extracellular  
22  
23 10 phosphate levels. *Applied and Environmental Microbiology* **65**: 3780-3786.  
24  
25  
26 11 Turner, B.L., Paphazy, M.J., Haygarth, P.M., Mckelvie, I.D. (2002) Inositol phosphates  
27  
28 12 in the environment. *Philosophical Transactions of the Royal Society B:*  
29  
30 13 *Biological Sciences* **357**: 449-469.  
31  
32  
33  
34 14 Ushasree, M.V., Shyam, K., Vidya, J., Pandey, A. (2017) Microbial phytase: Impact of  
35  
36 15 advances in genetic engineering in revolutionizing its properties and  
37  
38 16 applications. *Bioresource Technology* **245**: 1790-1799.  
39  
40  
41 17 Whitfield, H., Riley, A.M., Diogenous, S., Godage, H.Y., Potter, B.V.L., Brearley, C.A.  
42  
43 18 (2018) Simple synthesis of (<sup>32</sup>P)-labelled inositol hexakisphosphates for study  
44  
45 19 of phosphate transformations. *Plant Soil* **427**: 149-161.  
46  
47  
48 20 Wu, T.H., Chen, C.C., Cheng, Y.S., Ko, T.P., Lin, C.Y., Lai, H.L. *et al.* (2014) Improving  
49  
50 21 specific activity and thermostability of *Escherichia coli* phytase by structure-  
51  
52 22 based rational design. *J Biotechnol* **175**: 1-6.  
53  
54  
55  
56  
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56  
57  
58  
59  
60

1 Yao, Q., Li, Z., Song, Y., Wright, S.J., Guo, X., Tringe, S.G. *et al.* (2018) Community  
2 proteogenomics reveals the systemic impact of phosphorus availability on  
3 microbial functions in tropical soil. *Nat Ecol Evol* **2**(3): 499-509.  
4  
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4 2 Figure 1. HPLC analysis of the inositol phosphate content of zones of agar of PSM-  
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7 3 grown bacteria. A) *Bacillus subtilis* ESKAPE strain; B) *Escherichia coli*-pDES17-Btminpp  
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9 4 and C) *Pseudomonas putida* P450. A-C, non-cleared agar, grey lines; cleared agar,  
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11 5 black lines.  
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16 7 Figure 2. HPLC analysis of phytate degradation in liquid media of a soil culture.  
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18 8 A) day 0, shows minor impurities in the phytate substrate; B) by day 5, six InsP<sub>4</sub>  
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20 9 intermediates can be identified. Inset (A, B) shows traces expanded. C) a set of InsP  
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23 10 standards prepared by acid-reflux of phytate: the peaks identified are 1: InsP<sub>6</sub>, 2:  
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25 11 InsP<sub>5</sub> [2-OH], 3: InsP<sub>5</sub> [1/3-OH], 4: InsP<sub>5</sub> [4/6-OH], 5: InsP<sub>5</sub> [5-OH], 6: InsP<sub>4</sub>  
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27 12 (1456/3456), 7: InsP<sub>4</sub>(2456), 8: InsP<sub>4</sub> (1256/2345), 9: InsP<sub>4</sub> (1345/1356), 10: InsP<sub>4</sub>  
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29 13 (1245,2356), 11: InsP<sub>4</sub> (1234/1236), 12: InsP<sub>4</sub> (1246), 13: InsP<sub>3</sub>, 14: InsP<sub>1</sub>/P<sub>i</sub>.  
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36 15 Figure 3. HPLC analysis of phytate degradation by five different soil matrices. A)  
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38 16 Levington compost F2, and B) Church Farm were obtained in-house, C) Arable, D)  
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40 17 Bare Fallow and E) Broadbalk were obtained from Rothamsted Research long-term  
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42 18 field experiments. For E, the soil suspension was supplemented with additional  
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44 19 phytate. The traces are offset on the Y-scale. Black lines, day 0 (A,B), day 1 (C,D) or  
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46 20 day 3 (E). Dark grey lines, day 2 (A-D), day 7 (E). Light grey line, day 8 (E).  
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53 22 Figure 4. HPLC analysis of example phytate-degrading isolates. A) isolate AC1-2  
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55 23 (*Acinetobacter*) from agricultural soil and B) isolate CH-10-6-4 (*Buttiauxella*) from  
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57 24 Church Farm. Grey lines, day 0; black lines, day 2.  
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2 1 Figure 5. Phylogram of thirty-one Histidine Acid Phosphatases and twenty-seven  
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4 2 Multiple Inositol Polyphosphate Phosphatases (MINPP) showing the evolutionary  
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6 3 differences between the two sets of genes. The *Acinetobacter* sp. gene sequenced  
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8 4 (JABFFO000000000) is highlighted in blue. The *Buttiauxella* strain highlighted in red  
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11 5 is a species similar to that identified by 16s RNA sequencing of CH- CH-10-6-4  
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13 6 (accession MT680195).  
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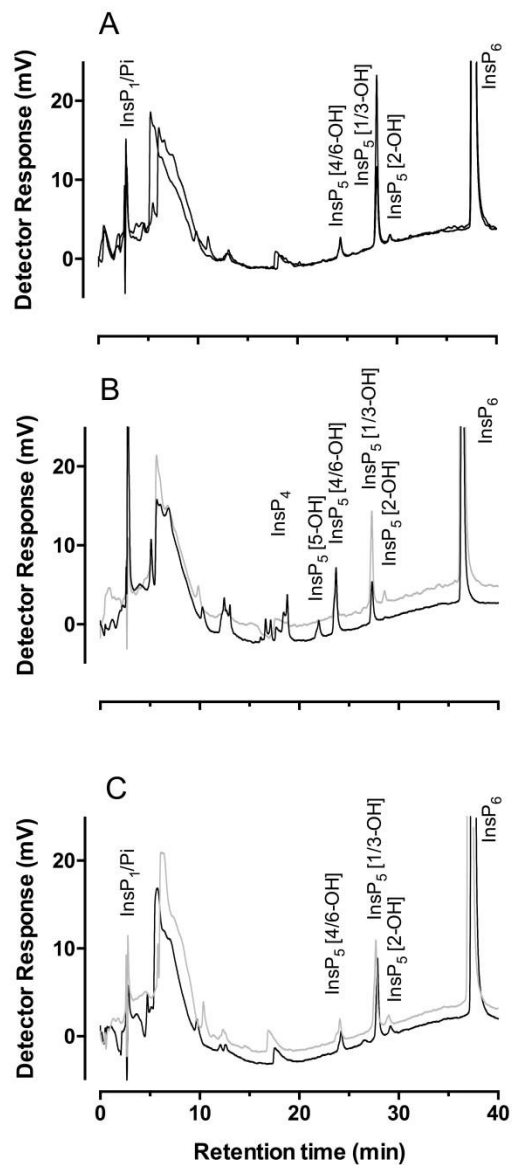


Figure 1. HPLC analysis of the inositol phosphate content of zones of agar of PSM-grown bacteria. A) *Bacillus subtilis* ESKAPE strain; B) *Escherichia coli*-pDES17-*Btminpp* and C) *Pseudomonas putida* P450. A-C, non-cleared agar, grey lines; cleared agar, black lines.

99x221mm (600 x 600 DPI)

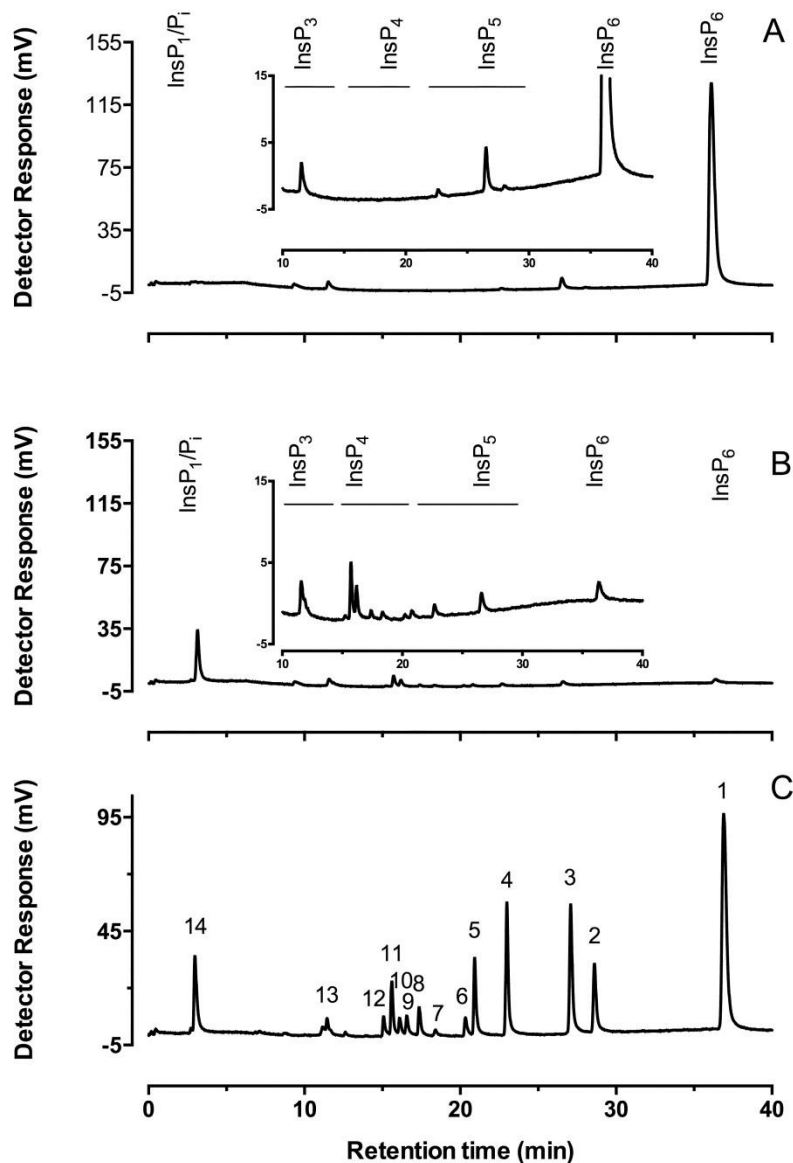


Figure 2. HPLC analysis of phytate degradation in liquid media of a soil culture.

A) day 0, shows minor impurities in the phytate substrate; B) by day 5, six InsP<sub>4</sub> intermediates can be identified. Inset (A, B) shows traces expanded. C) a set of InsP standards prepared by acid-reflux of phytate: the peaks identified are 1: InsP<sub>6</sub>, 2: InsP<sub>5</sub> [2-OH], 3: InsP<sub>5</sub> [1/3-OH], 4: InsP<sub>5</sub> [4/6-OH], 5: InsP<sub>5</sub> [5-OH], 6: InsP<sub>4</sub> (1456/3456), 7: InsP<sub>4</sub> (2456), 8: InsP<sub>4</sub> (1256/2345), 9: InsP<sub>4</sub> (1345/1356), 10: InsP<sub>4</sub> (1245,2356), 11: InsP<sub>4</sub> (1234/1236), 12: InsP<sub>4</sub> (1246), 13: InsP<sub>3</sub>, 14: InsP<sub>1</sub>/P<sub>i</sub>.

125x181mm (600 x 600 DPI)

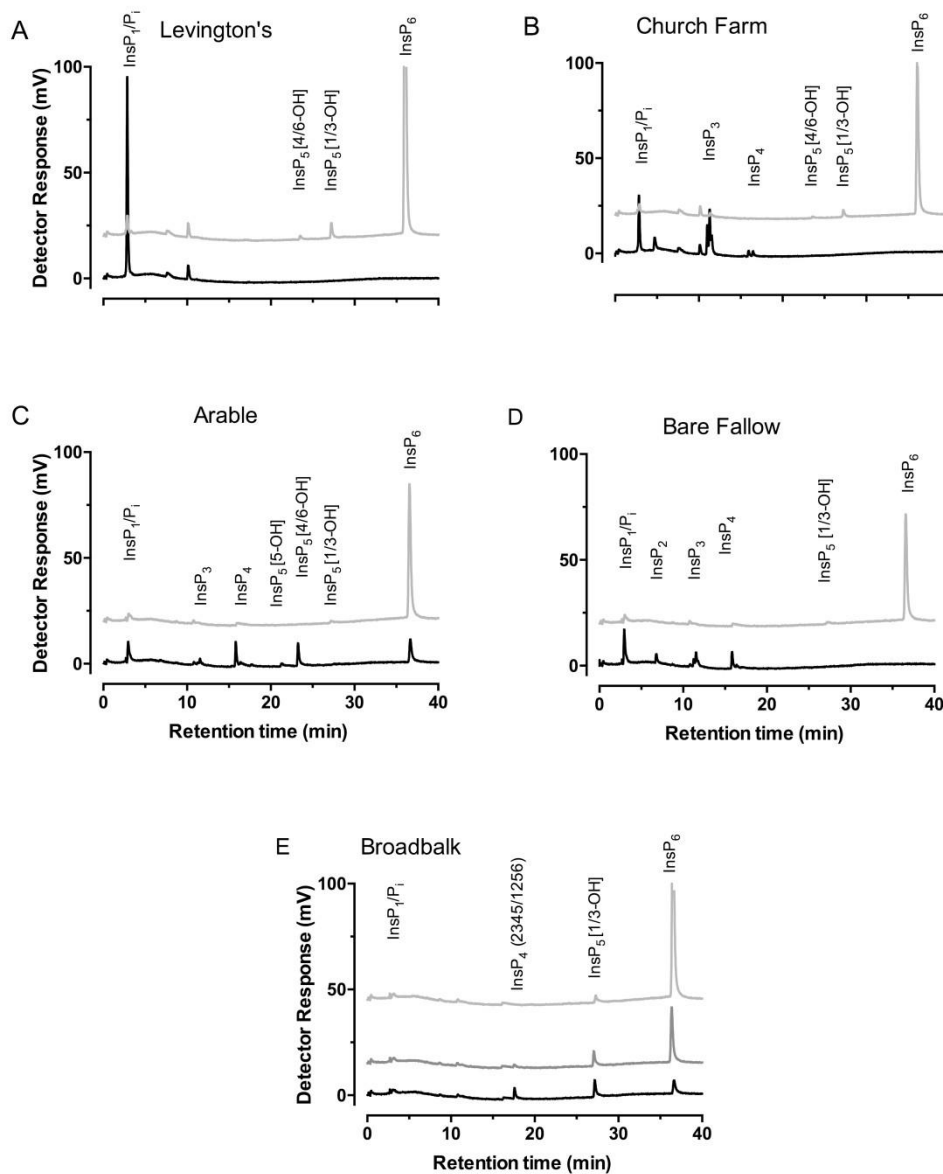


Figure 3. HPLC analysis of phytate degradation by five different soil matrices. A) Levington compost F2, and B) Church Farm were obtained in-house, C) Arable, D) Bare Fallow and E) Broadbalk were obtained from Rothamsted Research long-term field experiments. For E, the soil suspension was supplemented with additional phytate. The traces are offset on the Y-scale. Black lines, day 0 (A,B), day 1 (C,D) or day 3 (E). Dark grey lines, day 2 (A-D), day 7 (E). Light grey line, day 8 (E).

167x206mm (600 x 600 DPI)

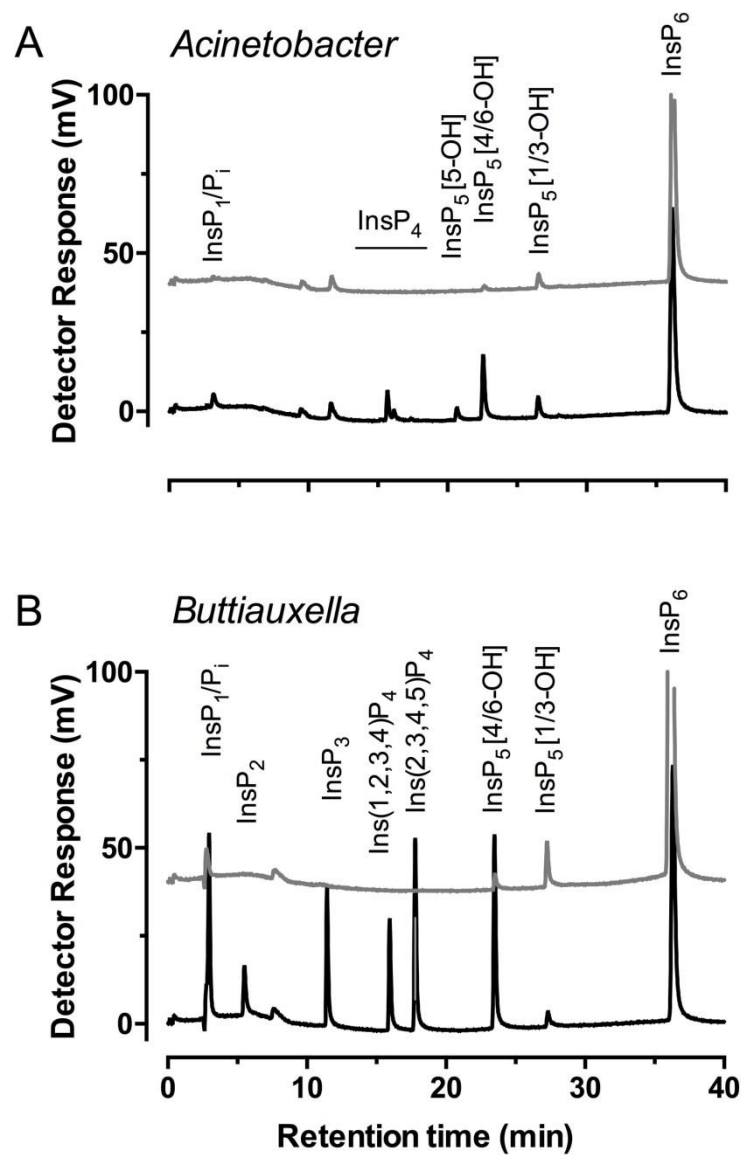


Figure 4. HPLC analysis of example phytate-degrading isolates. A) isolate AC1-2 (*Acinetobacter*) from agricultural soil and B) isolate CH-10-6-4 (*Buttiauxella*) from Church Farm. Grey lines, day 0; black lines, day 2.

137x213mm (300 x 300 DPI)

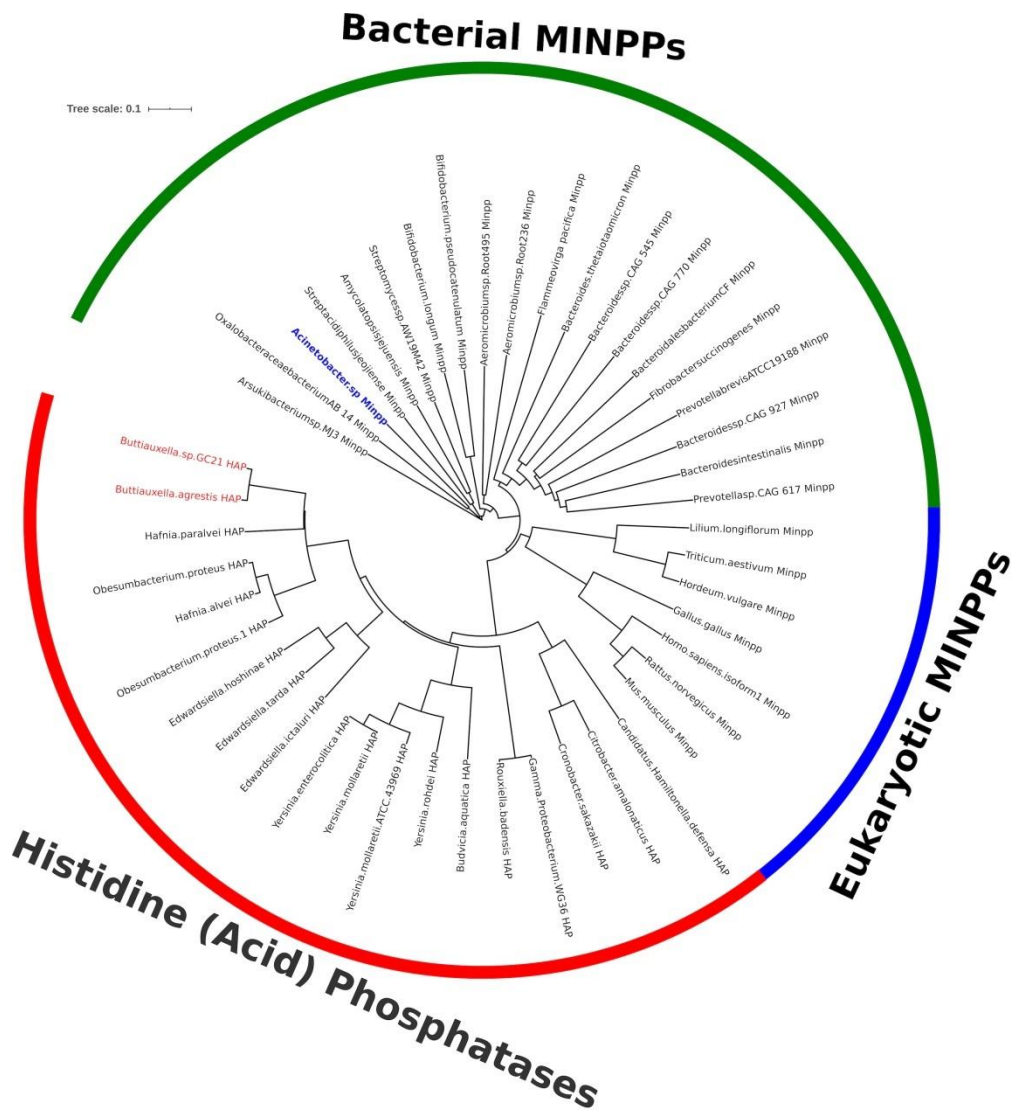


Figure 5. Phylogram of thirty-one Histidine Acid Phosphatases and twenty-seven Multiple Inositol Polyphosphate Phosphatases (MINPP) showing the evolutionary differences between the two sets of genes. The *Acinetobacter* sp. gene sequenced (JABFF000000000) is highlighted in blue. The *Buttiauxella* strain highlighted in red is a species similar to that identified by 16s RNA sequencing of CH-10-6-4 (accession MT680195).

119x131mm (635 x 635 DPI)