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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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11 5 Running title: MINPP activity in soil-dwelling *Acinetobacter*
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43 18 Keywords: HPLC-based screening; culture-dependent isolation; phytase; histidine
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45 19 acid phosphatase; multiple inositol phosphate phosphatase; soil phytate
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47 20

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.

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19 Introduction

20 There are four forms of phytic acid (inositol hexakisphosphate, InsP₆) 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 (InsP₆) 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*[®], 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, β -propeller phytases
18 (β 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
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19 9 substantial mole fractions of lower inositol phosphate and inorganic phosphate
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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 InsP_5 but not 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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38 16 The issue of substrate quality is relatively solvable; purification of InsP_6 is well
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40 17 described (Cosgrove, 1980; Dorsch *et al.*, 2003; Madsen *et al.*, 2019), but rarely
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42 18 discussed in screening for phytases. "Phytase specific media" (PSM) (Howson &
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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
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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
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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 β PPHy) and *Pseudomonas putida*
2 J450 (predicted to contain β 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.

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13 All profiles from the 'non-cleared' zones show the predominant peak of InsP_6 with a
14 retention time of c. 37 minutes and a smaller peak of 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' 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 InsP_6 degradation with a small
19 increase in InsP_5 [1/3-OH], and a concomitant increase in Pi. In this experiment
20 much of the InsP_6 remained. The InsP_5 [1/3-OH] peak is the expected product of the
21 known InsP_6 D-3-phosphatase activity of the β 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 InsP_5 ,
24 InsP_4 and 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.
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4 Finally, the *P. putida* strain (Figure 1C) showed little difference in the profile of
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7 'cleared' vs. 'non-cleared' agar despite the known InsP₆ D-3-phosphatase activity of
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10 other *Pseudomonas* sp. (Cosgrove *et al.*, 1970; Irving and Cosgrove, 1972).
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14 Collectively, these comparisons demonstrate that zone clearing without careful
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17 normalization is a poor assay for phytate degradation even of well-characterized
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20 organisms. It does illustrate however that HPLC can be combined with media-based
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23 culture and extraction of agar for testing of phytate degradation to provide high
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26 sensitivity and diagnostic analysis of the likely enzyme activity, by the simple
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29 expedient of observation of the occurrence of InsP peaks not present in 'non-
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32 cleared' regions of agar plates.
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34 Assay of phytate degradation by mixed population soil cultures

35 Phytate degradation may also be demonstrated with mixed cultures that might
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38 ordinarily be subjected to standard dilution and culture techniques for
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41 discrimination of individual isolates. In Figure 2 we show the result of mixing soil
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44 with minimal medium containing InsP₆ as the sole phosphate source. The soil was
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47 untilled (for the season) agricultural soil from Fakenham, Norfolk, UK, which we used
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50 to first test the technique. In this experiment, this agricultural soil was incubated
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53 with shaking at 30 °C. Degradation of InsP₆ was observed initially on day 3; by day 5
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56 less than 5% of starting InsP₆ remained, consistent with the accumulation of Pi,
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59 which co-elutes with InsP₁ on this column-gradient method. The generation of
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multiple inositol phosphate peaks at all stages of dephosphorylation (InsP₅, InsP₄,

1 InsP₃ and InsP₂) 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 InsP₅ and InsP₄ 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 β 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 β 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).

3
4 Figure 3.

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6 While degradation of phytate by some matrices, Levington's compost (Figure 3A),
7 Church Farm (Figure 3B) and Bare Fallow (Figure 3D) proceeded to completion or
8 close to it, indicated by predominant accumulations of Pi, other soils, which
9 presumably had less abundant or active cohorts of microbes, yielded diagnostic InsP_5
10 peaks in the timescale of the experiment. Of the Rothamsted soils, the Broadbalk
11 soil removed phytate from liquid media such that neither phytate nor lower inositol
12 phosphates were recovered, at day 0 (not shown). We attribute this to sorption of
13 phytate to soil particles as this has up to 35% clay content. Nevertheless, by
14 supplementing the soil/liquid mixture with 1 mM phytate we were subsequently
15 able to show that the soil and associated microorganisms were capable of processing
16 added phytate over 8 d (Figure 3E). For this soil, Pi did not accumulate in the
17 medium - suggesting that the microflora were efficiently scavenging the released
18 phosphate.

19 Classification of aggregate phytase activities of soil microbe populations

20 For phytases, the site of initial attack (Figure S1) represents one ontology of enzyme
21 classification. Enzyme Commission (EC) 3.1.3.26 - 4-phytase, defines enzymes that
22 remove phosphate from the 1D-4 position of *myo*-inositol hexakisphosphate
23 (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
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4 (Supplementary information) but the 3.1.3.26 signifier is commonly conflated with
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7 1D-6 phytase (1L-4 phytase) activity. EC 3.1.3.8 - 3-phytase, defines enzymes that
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10 remove phosphate from the 1D-3 position. This distinction makes no consideration
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12 of structural fold or reaction mechanisms.
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14 While bacterial and fungal [1D-] 3-phytases include histidine (acid) phosphatases
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17 with alpha-beta and alpha domain structure, Rossmann fold and characteristic
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19 reaction mechanism involving an attacking histidine nucleophile (His) and proton
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22 donating acidic amino acid (Oh *et al.*, 2004; Mullaney & Ullah, 2007), some [1D-] 3-
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25 phytases possess different structural folds. The β PPHy, exemplified by the enzyme
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28 from *B. amyloliquefaciens*, is a calcium-dependent metalloprotein with catalytic and
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31 structurally important bound Ca^{2+} ions (Shin *et al.*, 2001). Equally, histidine
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34 phosphatases include enzymes (phytases) that attack the 1D-6 position, exemplified
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37 by AppA from *E. coli* (Greiner *et al.*, 1993), *Buttiauxella* sp. (Cervin *et al.*, 2008),
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40 *Citrobacter* sp. (Kim *et al.*, 2003; Pontoppidan *et al.*, 2012) and *Hafnia alvei* (Ariza *et*
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43 *al.*, 2013). These are classified as EC 3.1.3.2 acid phosphatases. A comprehensive
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46 review of histidine phosphatases (Rigden, 2008) places fungal phytases and bacterial
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49 acid phosphatases in a branch of a superfamily of functionally diverse histidine
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52 phosphatases which include the enzymes phosphoglycerate mutase and fructose-
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55 2,6-bisphosphatase.
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58 Because simple HPLC resolves the two *meso*-compounds, InsP_5 [2-OH] and InsP_5 [5-
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61 OH] from the enantiomeric pairs InsP_5 [1/3-OH] and InsP_5 [4/6-OH] (Figure 2C) it is
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64 easy to distinguish between 4-phytases (EC 3.1.3.26) and 3-phytases (EC 3.1.3.8) or
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67 acid phosphatases (EC 3.1.3.2). For example, comparison of Highfield arable soil

1 (Figure 3C) - which yielded predominantly InsP₅ [4/6-OH] - with soil from a plot from
2 Broadbalk (Figure 3E) - which yielded InsP₅ [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 InsP₅ [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., InsP₅ [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 InsP₅ [4/6-OH] product, harboured by a soil earthworm castmicrobe
17 with similarity to *Sphingobium yanoikuyae* (Nasrabadi *et al.*, 2018).

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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 10⁶ 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 *Acinetobacter calcoaceticus* strain EB11, *Acinetobacter calcoaceticus* strain P19 and
2 that recovered from whole genome sequencing (JABFFO000000000) of the parent
3 isolate AC1-2. The 16S rRNA gene of strain CH-10-6-4 was identical to that in
4 *Buttiauxella agrestis* strain EB112, *Buttiauxella* sp. CL_136_AN_40 and *Buttiauxella*
5 sp. SA_136_AN_45 (MT450213-MT450215). To investigate the distribution of
6 different phytases in these two genera, BLAST searches were conducted using
7 ratified examples of each of five phytase families, restricting searches only to
8 *Acinetobacter* and *Buttiauxella* species (Table 1). For PAPHy, we followed Nasrabadi
9 et al. (2018) using *Lupinus luteus* (AJ505579) as query to blast *Sphingobium* spp.
10 genomes, returning hits with percentage identity 23–26% with E-value of 5^{-12} to 9^{-10} .
11 Subsequently, the full gene from *Sphingobium yanoikuyae* (CP060122) was used as
12 query of *Acinetobacter* and *Buttiauxella* spp. These searches indicated a divergent
13 distribution of phytase families between the two organisms. *Acinetobacter* species
14 were predominantly associated with MINPP and β PPhy, while histidine (acid)
15 phosphatase was the only phytase family associated with *Buttiauxella* species.

16
17 Table 1. Frequency of canonical phytase classes between referenced genomes
18 (Protein Blast of Non-Redundant Protein Sequences at NCBI) of *Acinetobacter* and
19 *Buttiauxella* spp.

20 The reference sequences used were: Multiple Inositol Polyphosphate Phosphatase
21 (MINPP), *Bacteroides thetaiotaomicron* (WP_009040027); Histidine (Acid)
22 Phosphatase (HAP), *Citrobacter amalonaticus* (DQ975370.1; β -propeller phytase
23 (β PPhy), *Bacillus amyloliquefaciens* (WP_013352583); Protein Tyrosine Phosphatase

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	β 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 β PPhy, or histidine (acid) phosphatase and β 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
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14 6 incubated with phytate and subjected to HPLC analysis (Figure 4A,B). This
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17 7 demonstrated that enzymes associated with AC1-2 are promiscuous in their site of
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19 8 initial attack on phytate substrate, yielding among InsP₅ isomers a dominant 4/6-OH
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21 9 peak, a smaller 5-OH peak and little to no detectable degradation at the 1/3-position
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23 10 (Figure 4A). Interestingly, strain CH-10-6-4 did not show any phytase activity in
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26 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
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41 17 initial position of attack on phytate, generating InsP₅ [4/6-OH] predominantly among
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43 18 InsP₅ products, consistent with the published properties of *Buttiauxella* phytase
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45 19 (Cervin *et al.*, 2008) and its industrial use (Ushasree *et al.*, 2017; Herrmann *et al.*,
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47 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 InsP₄

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56 23 intermediates: the *Acinetobacter* strain produced four InsP₄ intermediates, whilst

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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)P₄ and a minor peak with that of D/L-Ins(1,2,3,4)P₄.
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.

24

1 Figure 5.

2

3 Improved, predictive HPLC-based screening for phytases

4 The foregoing analyses highlight considerations that apply to culture-dependent

5 isolation of phytases, here from environmental samples. The methods described

6 overcome problems associated with the purity of phytate substrate (Madsen *et al.*,

7 2019) and 'zone-clearing' assays (Fredrikson *et al.*, 2002). Nevertheless, PSM can be

8 a useful media for obtaining a diverse set of bacteria (Greiner *et al.*, 1997;

9 Richardson & Hadobas, 1997; Kerovuo *et al.*, 1998) or for the screening of

10 engineered bacteria and plants (Shulse *et al.*, 2019).

11 Here, the opportunity to characterize enzyme activity of isolates before functional

12 cloning, expression, purification, subsequent verification of catalytic activity, is a

13 considerable shortcut that focuses attention among isolates on those with *bona fide*

14 phytase activity. Moreover, sequencing of the *Acinetobacter* and *Buttiauxella* strains

15 revealed the power of this HPLC-based screening strategy to illuminate phytase

16 diversity. The two different histidine phosphatases, MINPP and histidine (acid)

17 phosphatase, are typical of the families of enzymes identified in sequenced genera.

18 The assembled sequenced genome (JABFFO000000000) of the *Acinetobacter* strain

19 AC1-2 harbours a single histidine (acid) phosphatase of the MINPP class, rather than

20 a canonical histidine (acid) phosphatase.

21 The enzyme bears a hepta-peptide catalytic site sequence motif of RHGSRGL: RHG is

22 characteristic of the histidine phosphatase superfamily (Rigden, 2008), and the

23 proton donor motif is HAE, with glutamate replacing aspartate of the HD motif of

24 histidine (acid) phosphatases. AC1-2 MINPP is more closely related to eukaryotic,

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 NH₄Cl, 8.6 mM
15 NaCl, 1 mM MgSO₄, 0.1 mM CaCl₂, 1 mM succinate, 1mM glucose, 1mM sucrose,
16 1mM pyruvate, pH 7 and 1mM InsP₆. 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⁻¹) and with micronutrients: 10 µL (2 g nitriloacetic acid, 1 g MnSO₄.6H₂O,
20 0.8 g Fe(NH₄)₂(SO₄)₂, 0.2 g CoCl₂.6H₂O, 0.2 g ZnSO₄.7H₂O, 20 mg CuCl₂.2H₂O, 20mg
21 NiCl₂.6H₂O, Na₂MoO₄.2H₂O L⁻¹). The medium included 0.1-0.2 mg mL⁻¹ 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 dH₂O and 100 mg samples of agar
4 were extracted with 400 μ L 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 μ m 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 μ L 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 μ m pore
13 PTFE syringe filter (Kinesis, UK), centrifuged again and an aliquot (200 μ L) 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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2 1 Acknowledgments
3

4 2 GDR was funded by Natural Environment Research Council (NERC) PhD studentships
5
6 3 (NERC Doctoral Training Programme grant NE/L002582/1) with support from AB
7
8 4 Vista. ALN was supported by the Soil to Nutrition strategic programme
9
10 5 (BBS/E/C/000I0310) of the Biotechnology and Biological Science Research Council
11
12 6 (BBSRC) and by the Achieving Sustainable Agricultural Systems research programme
13
14 7 of NERC/BBSRC (NE/N018125/1 LTS-M). Access to the Rothamsted Long-term
15
16 8 Experiments National Capability [Highfield and Broadbalk experiments] was
17
18 9 supported by the Lawes Agricultural Trust and by BBSRC (BBS/E/C/00005189 and
19
20 10 BBS/E/C/000J0300). MicrobesNG (<http://www.microbesng.uk>), were funded by
21
22 11 BBSRC (BB/L024209/1).
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32 13 Author Contributions
33

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,
37
38 16 curated data and wrote the manuscript. CAB secured funding, supervised
39
40 17 experiments, and wrote the manuscript.
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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 InsP₄
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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: InsP₆, 2:
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25 11 InsP₅ [2-OH], 3: InsP₅ [1/3-OH], 4: InsP₅ [4/6-OH], 5: InsP₅ [5-OH], 6: InsP₄
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27 12 (1456/3456), 7: InsP₄(2456), 8: InsP₄ (1256/2345), 9: InsP₄ (1345/1356), 10: InsP₄
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29 13 (1245,2356), 11: InsP₄ (1234/1236), 12: InsP₄ (1246), 13: InsP₃, 14: InsP₁/P_i.
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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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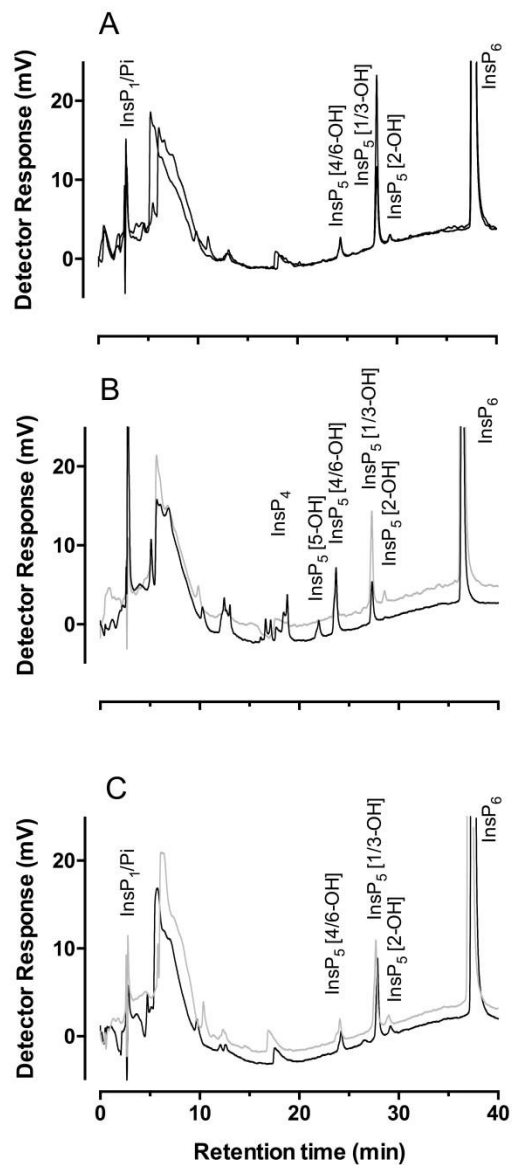


Figure 1. HPLC analysis of the inositol phosphate content of zones of agar of PSM-grown bacteria. A) *Bacillus subtilis* ESKAPE strain; B) *Escherichia coli*-pDES17-*Btminpp* and C) *Pseudomonas putida* P450. A-C, non-cleared agar, grey lines; cleared agar, black lines.

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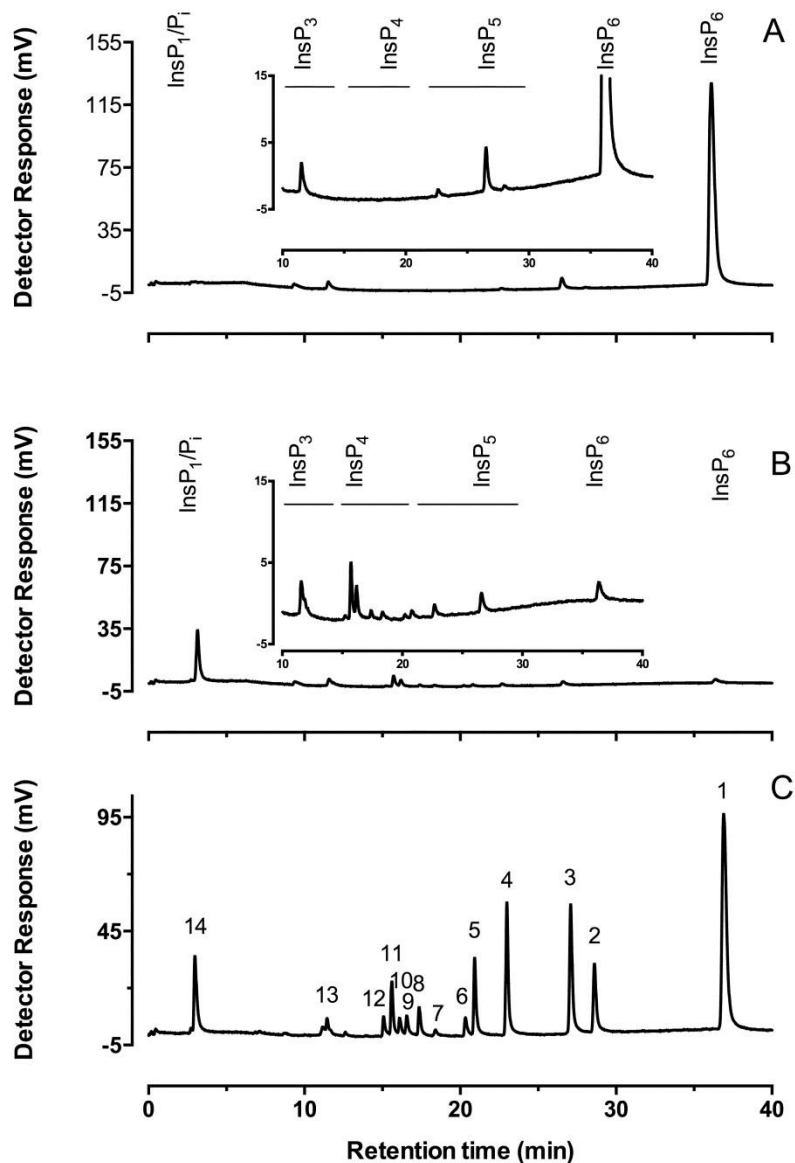


Figure 2. HPLC analysis of phytate degradation in liquid media of a soil culture.

A) day 0, shows minor impurities in the phytate substrate; B) by day 5, six InsP_4 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_6 , 2: InsP_5 [2-OH], 3: InsP_5 [1/3-OH], 4: InsP_5 [4/6-OH], 5: InsP_5 [5-OH], 6: InsP_4 (1456/3456), 7: InsP_4 (2456), 8: InsP_4 (1256/2345), 9: InsP_4 (1345/1356), 10: InsP_4 (1245,2356), 11: InsP_4 (1234/1236), 12: InsP_4 (1246), 13: InsP_3 , 14: InsP_1/P_i .

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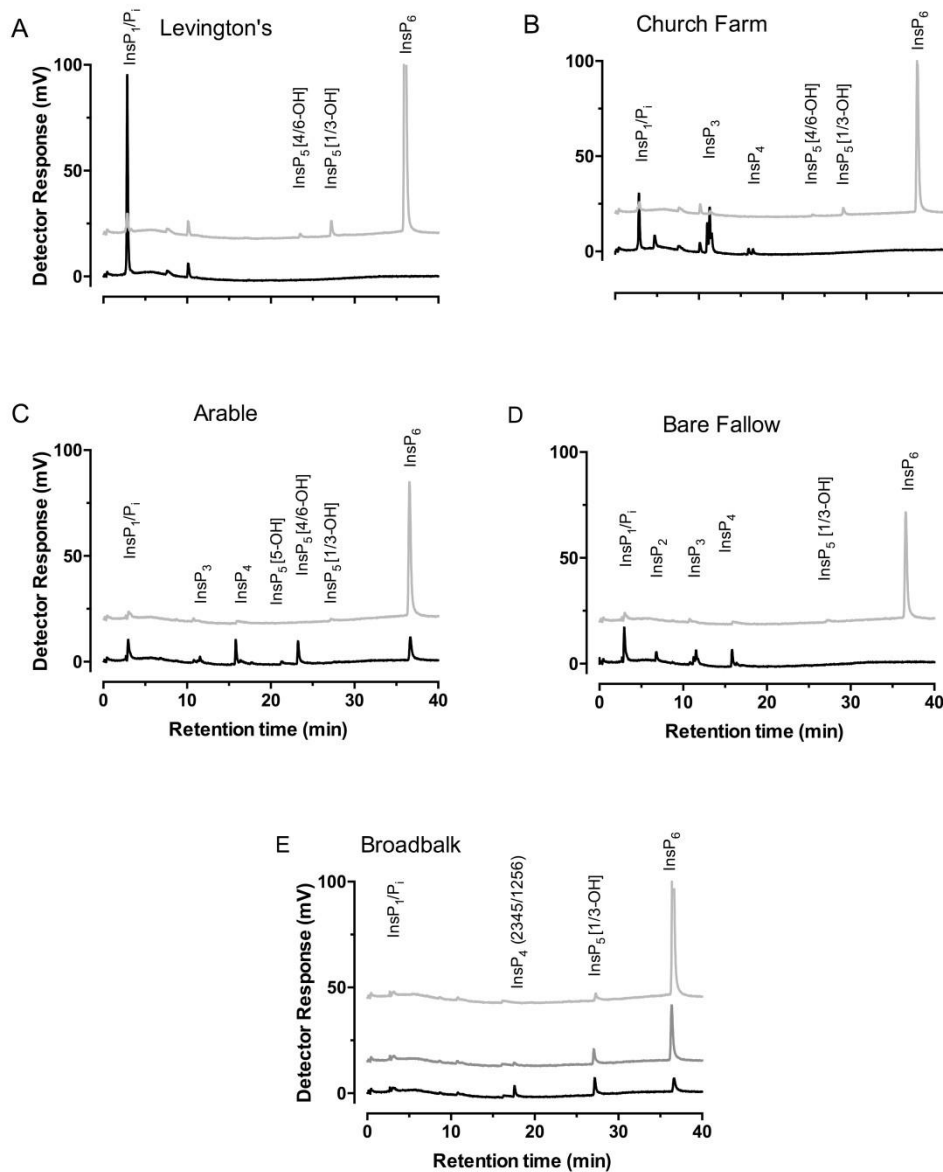


Figure 3. HPLC analysis of phytate degradation by five different soil matrices. A) Levington compost F2, and B) Church Farm were obtained in-house, C) Arable, D) Bare Fallow and E) Broadbalk were obtained from Rothamsted Research long-term field experiments. For E, the soil suspension was supplemented 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).

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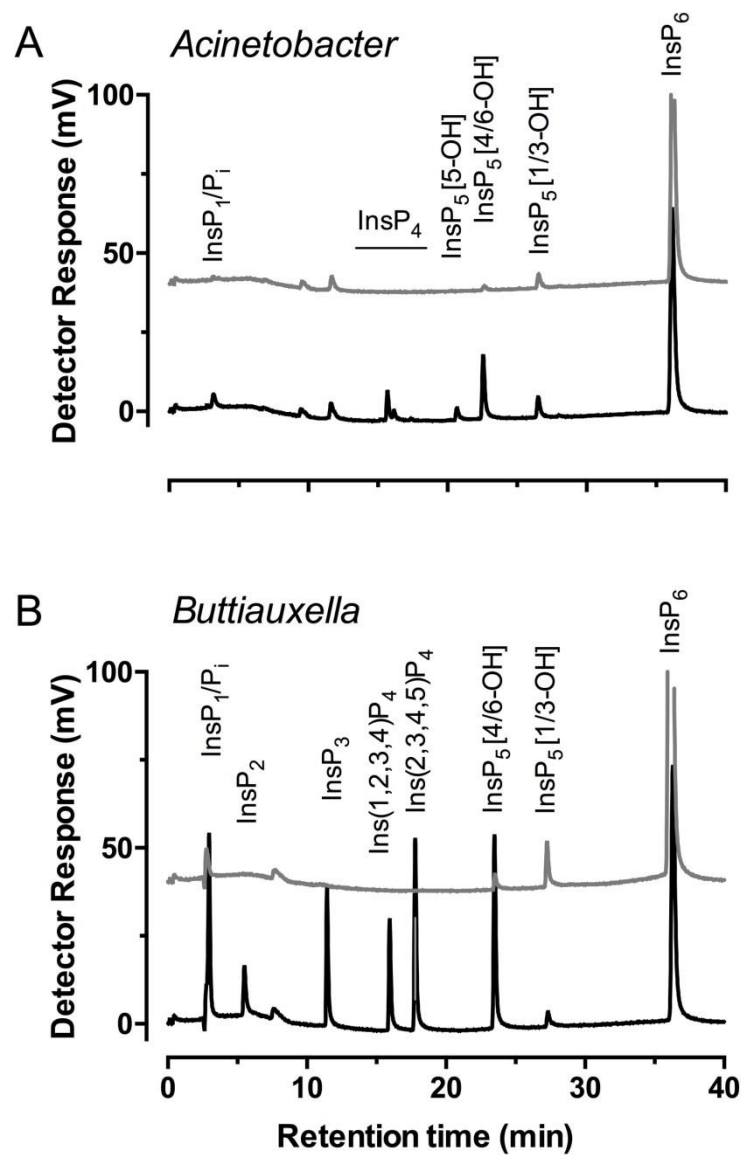


Figure 4. HPLC analysis of example phytate-degrading isolates. A) isolate AC1-2 (*Acinetobacter*) from agricultural soil and B) isolate CH-10-6-4 (*Buttiauxella*) from Church Farm. Grey lines, day 0; black lines, day 2.

137x213mm (300 x 300 DPI)

