Transcription of macromolecular N degrading genes provides a window into organic nitrogen decomposition in soil

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Transcription of macromolecular N degrading genes provides a window into organic nitrogen decomposition in soil

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Abstract

Background: Nitrogen (N) is commonly limiting in soil in part because most N is present in macromolecular organic compounds and not directly available to plants. Using extracellular enzymes, the soil microbial community present near roots (rhizosphere) is largely responsible for transforming organic substrates, which yields nitrogen in forms that are plant-available. Microbial genetic potential (genes) and the cycling of inorganic nitrogen pools have been extensively documented and widely explored in rhizospere soil. But there is scarce information on microbial gene expression for macromolecular nitrogen decomposition and how it relates in space and time to life stages of plant roots. Here we use a suite of time-resolved metatranscriptomes from rhizosphere and bulk soil to follow bacterial and fungal extracellular protease and chitinase expression during rhizosphere aging. We also explore the effect of adding plant root litter as a distinct source of macromolecular carbon and nitrogen.

Results: Expression of extracellular proteases in rhizosphere soil increased with plant growth in the absence of litter, whereas chitinase (*chit1*) was highly upregulated in the detritusphere. Structural groups of proteases were dominated by serineproteases, despite the higher abundance of metalloproteases previously observed in soil and aquatic systems. Extracellular proteases of Betaprotebacteria were more highly expressed in the rhizosphere, whereas those of Deltaroteobacteria and Fungi responded strongly to the presence of litter. We identified distinct functional guilds of taxa specializing in decomposition of proteins in the rhizosphere, detritusphere and in the vicinity of aging roots. We also identify a sub-guild that appears to specialize in protein decomposition in the presence of growing roots as well as litter that

increases its activity in aging rhizosphere soil, which may be particularly N limited. Taxonomic membership of these guilds partially overlapped with functional guilds identified by carbon decomposing enzymes, making up multifunctional guilds.

Conclusions: We found organic N decomposers have distinct spatiotemporal preferences that are selected during plant growth. Rhizosphere microbes which could potentially benefit plants by degrading macromolecular nitrogen were not comparatively active in bulk soil, even in the presence of root litter, implying that they are enhanced near plant roots and less competitive in bulk soils.

Keywords

Rhizosphere, microbiome, protease, chitinase, functional guilds, metatranscriptome, gene expression, litter

Background

Plants are commonly limited by nitrogen (N) in temperate soils since their access to the largest soil N pools is constrained by the activity of microbes responsible for mineralization of macromolecular nitrogen and N_2 fixation [1–3]. To a large degree, plants depend on microbial degradation of macromolecular forms of N such as proteins and chitin [4–6] and can potentially increase N availability by releasing exudates that stimulate microbial turnover of organic N pools (via 'priming') [7–10]. Depolymerization of high molecular weight detrital organic N is one of the rate-limiting steps in soil N mineralization [11,12] and depends on the activity of extracellular enzymes such as lysozyme, protease, chitinase, nuclease and urease. The resulting N monomers can be further broken down to NH_4 or in some cases taken up directly by the plant [13]. While the genetic potential and bulk-scale activity of extracellular N degrading enzymes is well established, the spatial and temporal dynamics of gene expression underpinning N mineralization are ill-defined. In addition, soil microorganisms capable of degrading macromolecular N are highly diverse [14], and it is unclear when and where specific taxonomic groups are active [15].

Patterns and controls of soil N depolymerization and mineralization may be highly dependent on the habitat (e.g., rhizosphere, detritusphere), since availability of organic-N substrates varies in both time and space, as does the prevalence of fungi, fauna and bacterial degraders, which can enable or limit N-cycling. In the rhizosphere, organic N is available as amino acids, nucleotides, niacin, and choline (derived from plant exudates [10]), lignoproteins and aromatics from sloughed off cells, nucleic acids, and microbial cell wall amino sugar

polymers (N-acetylglucosamine, N-acetylmuramic acid) derived from the bloom of cells that develops as roots grow [15]. Moreover, root exudates can drive destabilization of mineral-associated organic matter (MAOM), creating an additional source of macromolecular N [13]. Prior work indicates that root exudates can increase degradation of soil organic matter by up to 380% [16]. Chitinase and protease enzyme activities were shown to be significantly upregulated in the rhizosphere of wild oatgrass (*Avena spp.*), and N mineralization was highly dependent on quorum sensing [17]. This contrasts with the detritusphere, which has a relatively higher proportion of aromatic N and lignoproteins. Protease enzyme activity has been shown to be enhanced by litter addition [18] and root exudates [19], as well as vary with type of plant litter [20,21]. Obviously, these spatial habitats (rhizosphere and detritosphere) can overlap—as when a root initially grows through bulk soil, or when a growing root begins to age and resemble detritus.

Soil N pools and by proxy, N mineralization activity, change with time [22] in part due to the succession of both exudate quality and microbial communities [10,23]. At the bulk soil level, activity of extracellular proteases is thought to drive soil N cycling [12], but what controls this activity is not always clear [24]. Subtle distinctions caused by time and soil habitat are likely masked because most studies are conducted on whole soils, which contain a mixture of rhizosphere, detritusphere and bulk regions. In grassland soils, the rhizosphere is a particularly critical hotspot for microbial biomass and activity due to rapid recycling of root exudation and debris, and functions as a quasi-digestive system decomposing molecules inaccessible to plants. However, root exudation, which serves as a carbon source for rhizosphere bacteria, declines near older root sections [25]. Therefore, it has been hypothesized that bacteria in a mature rhizosphere environment may be forced to target less labile, higher C:N sources of organic N such as plant

litter [26]. Indeed, it has been shown that the mature *Avena* sp. rhizosphere has an increased organic N demand relative to the young rhizosphere, where the mature rhizosphere has higher rates of gross nitrogen mineralization and ammonia consumption [28].

Of the vast number of microorganisms that exist in a soil, at any given time, only a small proportion (e.g., 8%) are active in the rhizosphere [15,17,27,28]. Gene expression studies show that soil bacteria, in the rhizosphere and in the presence of plant litter, form functional guilds [29–31]. However, there has been little focus on guilds based on macromolecular N degradation. To maintain cellular C:N stoichiometry, microbial carbon acquisition must be accompanied by N uptake. Therefore, we postulate that an analogous functional guild organization occurs in expression of extracellular proteases as with CAZy guilds, as exoproteases are diverse enzymes that target a variety of substrates and have also been shown to be influenced by soil characteristics [32–34]. However, while a general balance of C and N degradation is expected to maintain cellular C:N, it does not follow that the same taxa should be involved in both processes. The capacity to utilize specific C and N forms is taxon-specific; e.g., some bulk soil bacteria may degrade carbohydrates as a C source and take up inorganic N, whereas some rhizosphere bacteria may take up simple root exudates as a C source but degrade proteins as an N source.

We hypothesized that community-wide expression and taxon-specific differential expression of extracellular proteases and chitinases differed in rhizosphere vs. bulk soil during root growth and that expression profiles would be altered by root litter amendments. To address that hypothesis, we used metatranscriptomes collected over time from rhizosphere microcosms, using a robust, well-developed experimental approach [17,23,26,29] with a well-characterized annual grassland soil from Northern California. Mature wild oat grass *Avena fatua* [22] plants were monitored for 3 weeks to determine the role of diverse indigenous rhizosphere bacteria in

macromolecular nitrogen degradation by extracellular proteases and chitinases throughout root growth and aging. In addition, we compared functional guilds based on extracellular protease expression with guilds defined by expression of carbohydrate active enzymes (CAZy) capable of degrading root litter.

Methods

Experimental design, sample collection and sequencing

The experimental design, sample collection and sequence data processing are described in detail in [29]. Briefly, common wild oat *Avena fatua* was grown for six weeks in rhizobox microcosms containing soil from the Hopland Research and Extension Center (HREC) in northern California, a Bearwallow–Hellman loam (pH 5.6, 2% total C) packed at field bulk density. Roots were then allowed to grow into a sidecar soil region with a transparent wall, where the root growth timeline was marked at 3 days, 6 days, 12 days and 22 days. In half of the sidecars, the soil was amended with dried *A. fatua* root detritus (C:N = 13.4) chopped to 1 mm. Bulk soil bags, inaccessible to roots, were placed in each sidecar; a bulk soil treatment amended with root detritus was also included. At each timepoint, three replicate microcosms were destructively harvested for paired rhizosphere and bulk soil, both detritus amended and unamended, yielding a total of 48 samples representing four treatments at four timepoints: rhizosphere, rhizosphere + litter, bulk, and bulk + litter.

Harvested soil (1 g) was placed immediately in Lifeguard Soil Preservation Reagent (MoBio) and processed according to the company protocol. Roots and supernatant were

removed, and the soil was stored in -80°C. DNA and RNA were co-extracted using a phenol-chloroform procedure [35,36] and separated with an AllPrep kit (Qiagen). RNA was DNase treated (TURBO DNase, Thermo-Fisher Scientific), depleted in ribosomal RNA (Ribo-Zero rRNA Removal Kit, Epicentre) and reverse transcribed into cDNA. Metatranscriptomes were sequenced for 48 samples on an Illumina HiSeq 2000 2x150 (TruSeq SBS v3) at the Joint Genome Institute (JGI), in Walnut Creek CA.

Expression of chitinase and extracellular protease genes identified in assembled metatranscriptomes

Raw reads were quality-trimmed (Q20) and rRNA and tRNA reads were removed. Library size, evenness, richness and Shannon diversity were comparable between experimental groups with a mean library size of 43 M paired end reads after filtering. Quality-controlled metatranscriptomic reads were assembled into contigs within each sample [29]. Contigs smaller than 200bp were discarded and the remaining contigs from all samples were clustered at 99% identity with cd-hit-est keeping the longest sequence as the cluster representative [37]. Open reading frames (ORFs) were predicted using Prodigal [38]. Extracellular protease ORFs were identified by reciprocal BLAST to extracellular peptidases from the MEROPS database [14,39]. ORFs were assigned a peptidase group (serine-, metallo-, cysteine-peptidase and others) by their best reciprocal BLAST hit. Taxonomy was determined by best BLAST hit to the NCBI non-redundant database (NR, access date July 29th 2019). Chitinase ORFs were identified using six chitinase hidden Markov models (HMMs) from the Kyoto Encyclopedia of Genes and Genomes (KEGG): K01183, K03791, K13381, K17523, K17524 and K17525. Only the first three were

detected in our dataset. Reads were then mapped back to ORFs requiring minimum identity 95% and 75% breadth using bbmap [40]. Read counts were normalized using DESeq2 [41]. Heat maps of normalized counts were generated in R using the heatmap2 function in gplots [42]. Normalized transcript counts per gene per time point were compared between groups (rhizosphere, litter and litter-amended rhizosphere) using ANOVA and Tukey post-hoc test. Boxplots were generated in ggplot2 [43].

Expression of extracellular protease genes from a curated collection of Hopland-soil genomes

The quality-controlled reads were mapped using BBsplit [40] requiring 80% identity against a dereplicated reference database of 282 HREC soil genomes including isolates [10], single amplified genomes (SAGs) [29], metagenomic assembled genomes (MAGs) (NCBI PRJNA517182) and stable isotope probing MAGs (SIP-MAGs) [44]. On average, 12.3% (range 6.2-31.9%) of the reads per library mapped unambiguously to genomes from the reference database. This additional approach was taken to investigate gene expression within the context of a genome and to search for overlap in guild membership between extracellular protease defined guilds and previously defined CAZy guilds [29]. Three MAGs that were classified as unknown or domain bacteria in previous studies were assigned taxonomy using GTDB-Tk version 1.3.0 [45,46]. Verification of the taxonomic assignment of MAG Burkholderiales_62_29 was done using GToTree [47] with complete reference genomes of Betaproteobacteria from RefSeq (Feb 28, 2020).

Open reading frames (ORFs) were predicted in all genomes using prodigal [38] and annotated using KEGG [48] and ggKbase (http://ggkbase.berkeley.edu). Extracellular proteases,

which do not have hidden Markov models (HMMs) capable of separating them from intracellular proteases, were identified by gene nucleotide identity of at least 90% and coverage of at least 60% to *de novo* assembled ORFs of extracellular protease from the metatranscriptomes. Gene counts were identified using Rsubread featureCounts [49].

Differential expression of chitinases in the genome collection was already performed in a previous publication on expression of carbohydrate active enzymes (CAZy) and was therefore not repeated here [29].

Statistical analyses

All features and their abundance (represented by metatranscriptomic read counts normalized to sequencing depth) were analyzed with DESeq2 [41] requiring p-value < 0.05 (adjusted for multiple comparisons). Ordination and visualization were conducted in R using ggplot2 [43] and vegan [50]; PERMANOVA (vegan function adonis) was used to detect significant treatment factors affecting expression of nitrogen depolymerization genes. We define 'guilds' as groups of taxa with similar gene upregulation patterns of extracellular proteases in both time and soil habitat. Guilds were assigned by hierarchical clustering based on differential expression of extracellular proteases compared to unamended bulk soil, generating four functional guilds: "Rhizosphere", "Detritusphere", "Aging root", and "Low response". Effects of the four experimental treatments (bulk, rhizosphere, litter and litter-amended rhizosphere) on protease gene expression were assessed by ANOVA (p adjusted for multiple comparisons), N=3 per timepoint.

Results

Expression of enzymes targeting macromolecular N

Multiple genes related to degradation of macromolecular N were identified in this experiment: extracellular nuclease (Xds), urease (UreABC), lysozyme, chitinase (chit1) and extracellular proteases. Expression of extracellular proteases was an order of magnitude higher than the extracellular N degrading enzymes (sup. fig. S1), and like chitinases, was affected by the presence of litter and roots. In contrast, extracellular nuclease (Xds), urease (UreABC) and lysozyme were not influenced by either living or decaying roots, thus, we chose to focus on extracellular proteases and chitinases.

Normalized transcript abundance for 4948 extracellular protease genes from bacteria (4846) and fungi (102) were quantified in the sampled soils. Overall, extracellular protease expression was significantly affected by time (3-way PERMANOVA, $F=2.8$, $p=0.038$), litter amendment ($F=118.9$, $p<0.001$) as well as interactions between time:treatment ($F=26.9$, $p<0.001$), time:location ($F=7.7$, $p<0.001$) and location:treatment ($F=15.5$, $p<0.001$). Expression in unamended soil (no litter) generally increased over time (fig. 1A), whereas litter-amended soil had initially high expression that then decreased and leveled off over time (fig. 1B).

The 73 distinct chitinase transcripts were expressed at a substantially lower level than the extracellular proteases. The expression of the *chit1* gene increased over time in litter-amended soils and was higher than in the non-litter treatments (3-way PERMANOVA, $p<0.001$) (fig. 1C). Expression of fungal chitinases was lower than bacterial chitinases, and both were generally lower than eukaryotic chitinases (fig. 1D). Transcripts for chitinase KEGG orthologs *CHI3L1_2*, *CHI3L1_4* and *chiA* were not detected at all and the putative chitinase K03791 had low

expression and no significant effects with time or treatment (data not shown). Chitinase CHID1 was detected but was most closely related to plants and therefore disregarded. A companion study analyzed differential expression of chitinases within our curated genome collection and while only one MAG was found to upregulate chitinase, it did so in the presence of litter [29].

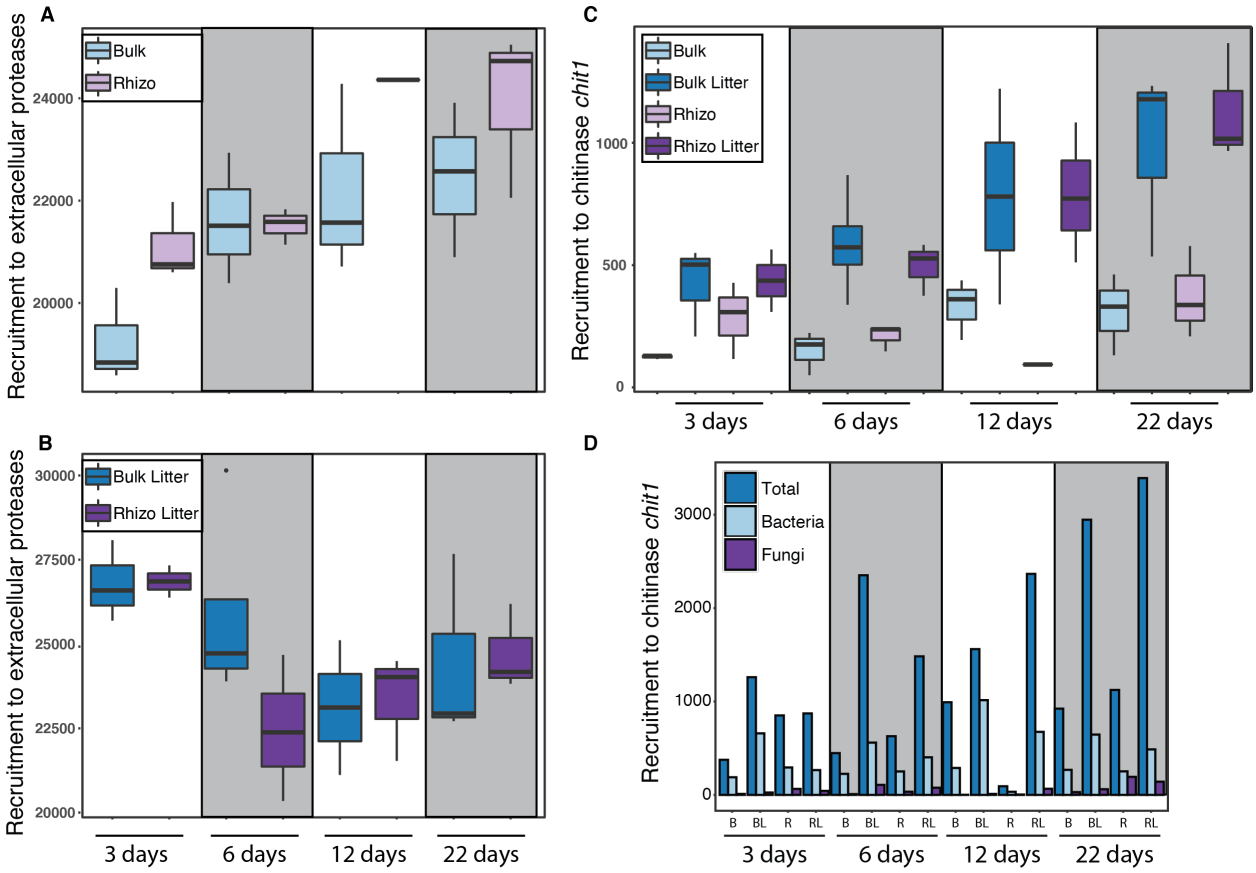


Figure 1: Aggregated expression of extracellular proteases in bulk and rhizosphere soils from common wild oat *Avena fatua* microcosms, grown without root litter amendment (A), or with litter amendment (B); expression of chitinase gene *chit1* (C) and mean counts of chitinases at the domain level (D) for unamended bulk soil (B), litter-amended bulk soil (BL), unamended rhizosphere (R) and litter-amended rhizosphere (RL). Boxplots represent 75% of the data,

whiskers denote 90% of the data and dots (e.g. panel A bulk litter 6 days) represent outliers. Note that the scale of the Y axis varies between panels.

Structural groups of extracellular proteases

Structural groups of extracellular proteases can be soil-specific and pH dependent [32]. The main groups of extracellular proteases found in our assembled metatranscriptomes were serine-, metallo- and cysteine-proteases (2679, 1949 and 496, respectively out of 5295 variants clustered at 99% identity). Aggregated expression patterns of all variants in each group reflected the same order (serine>metallo>cysteine). Expression of serine- was consistently higher than metallo-protease across all treatments (ANOVA $F=2392$, $p=0$) (fig. 2, sup. table S4). Expression of serine-proteases was also significantly greater in the presence of litter compared to no litter (diff=2337, $p<0.001$), but root litter amendment did not affect expression of metallo- or cysteine-proteases, and there was no significant effect of time or location on these structural groups (sup. table S4).

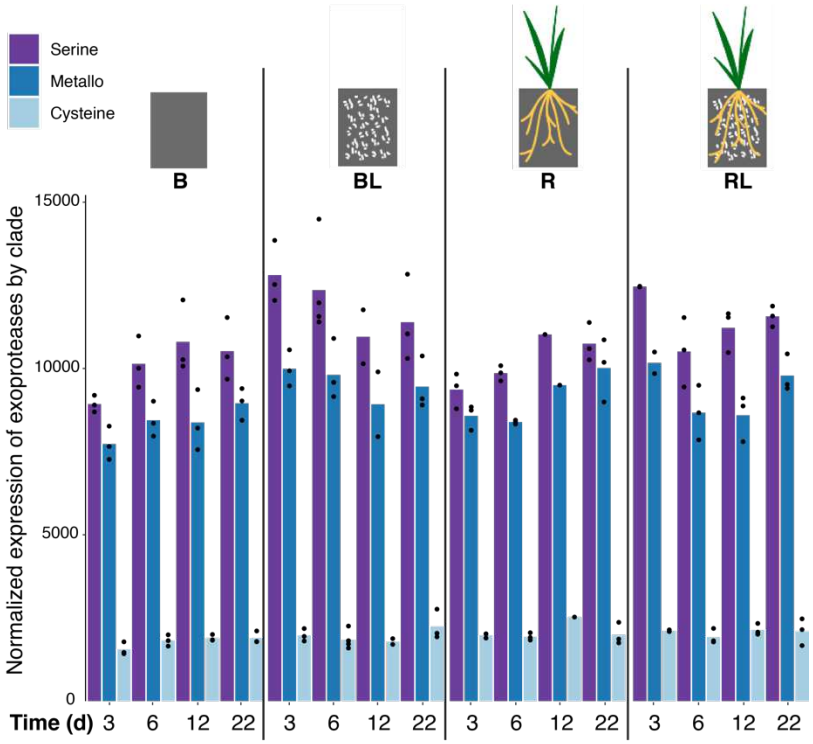


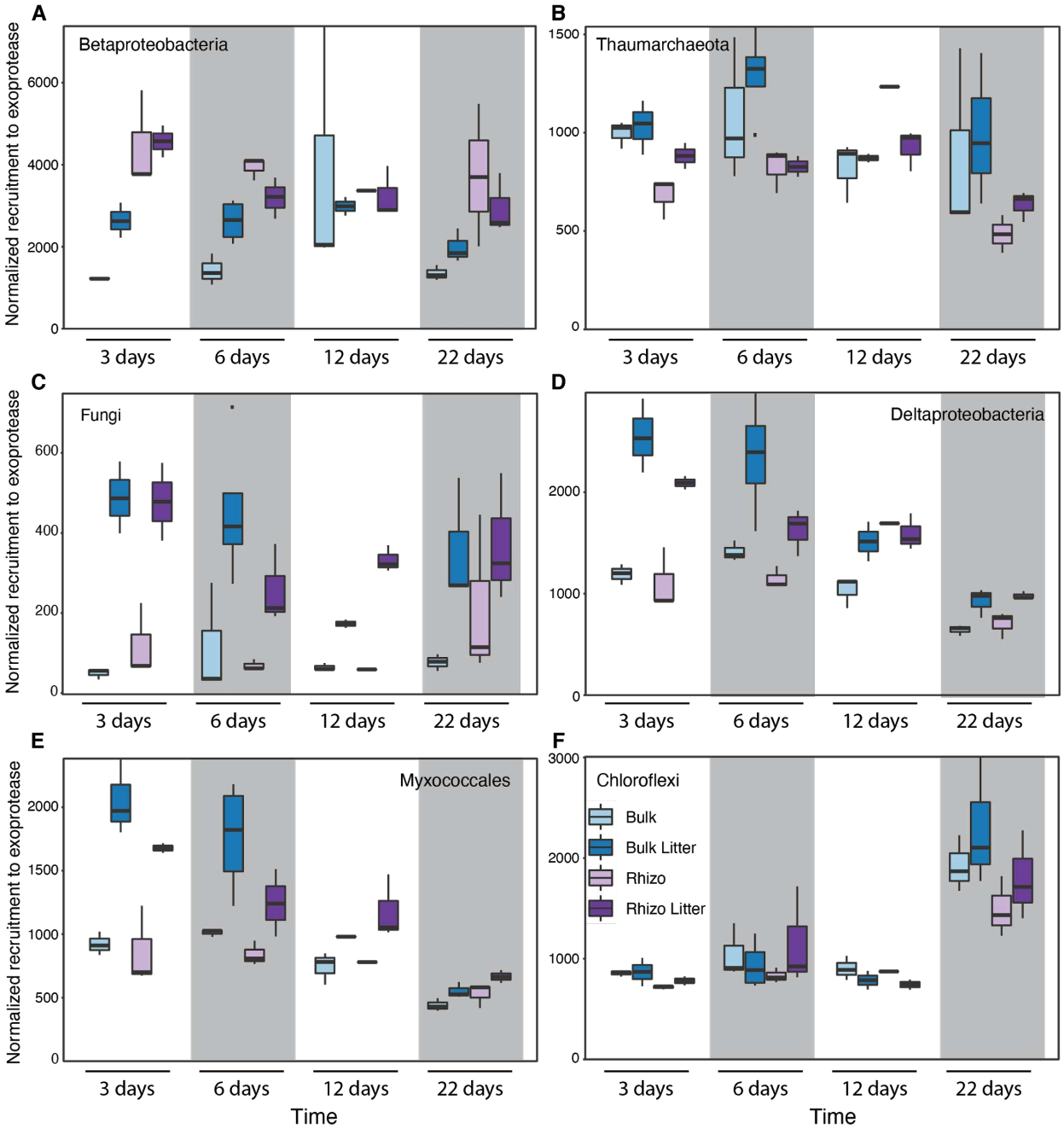
Figure 2: Aggregated normalized expression of extracellular protease structural groups over time in bulk, rhizosphere and root litter-amended soils. Normalized expression per replicate is plotted as dots on top of the bars. Experimental groups shown per time point are (left to right): unamended bulk soil (B), litter-amended bulk soil (BL), unamended rhizosphere (R) and litter-amended rhizosphere (RL). Extracellular protease groups are (left to right): serine-, metallo- and cysteine-protease.

Taxonomy of extracellular proteases

De novo assembled ORFs of extracellular proteases were taxonomically assigned by BLASTP best hit against the NCBI non-redundant database (NR). Since extracellular proteases are not marker genes, we considered taxonomic assignments only at the order level or higher,

with the exception of *Rhizobacter*, for which amino acid percent identity values were extremely high (84±8.4% amino acid identity).

In the rhizosphere, Betaproteobacteria exoproteases were significantly upregulated (ANOVA, $p<0.05$) (fig. 3A). Of 547 variants of exoproteases from this class, 442 were assigned as Burkholderiales. In contrast, at most timepoints, proteases of Cyanobacteria and Thaumarchaeota were significantly downregulated in the rhizosphere compared to bulk soil (fig. 3B; sup. table S1). In multiple other taxonomic groups, proteases were significantly upregulated only in the presence of litter: Fungi (fig. 3C), class Deltaproteobacteria (fig. 3D), as well as highly represented phyla Bacterioidetes and Verrucomicrobia (sup. fig. S2; sup. table S1) and classes Chitinophagia and Gammaproteobacteria (sup. table S2). Interestingly, exoprotease expression declined with time for the predatory bacteria Myxococcales (fig. 3E), Bdellovibrionales and Cytophagia, while clades such as Chloroflexi (fig. 3F) and Actinobacteria had increased exoprotease expression at the final sampling point. While the Actinobacteria and Acidobacteria had a high number of variants and high expression of exoproteases, we did not detect a significant effect of either time or soil habitat/ amendment. Normalized protease expression data are summarized by phylum (sup. fig. S2), class (sup. fig. S3) and order (sup. fig. S4), and ANOVA F and p values are in sup. table S1, sup. table S2 and sup. table S3 respectively.



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310

311 Figure 3: Expression of extracellular proteases over time for select taxonomic groups: (A)

312 Betaprotebacteria (n=547) were upregulated in the rhizosphere, (B) Thaumarchaeota (n=112)

313 were downregulated in the rhizosphere, (C) Fungi (n=99) were upregulated in the presence of

314 litter, (D) Detaproteobacteria (n=228) were upregulated in the presence of litter, (E)

Myxococcales (n=147) were upregulated in the presence of litter and decreased over time and (F) Chloroflexi (n=153) increased in the last time point. The legend in panel F applies to all panels. Note that as expression levels varied over taxonomic groups and to emphasize patterns, the y axes do not have the same scale.

Functional guilds

To define functional guilds with a population-centric analysis, we mapped transcriptome reads to a collection of 282 genomes and metagenome-assembled genomes generated from the same soil and site [29]. Read counts were used to determine differential expression compared to unamended bulk soil. Each genome contained multiple genes coding for extracellular proteases. Hierarchical clustering of the mean differential expression of extracellular protease genes within each genome was used to define four functional guilds: “rhizosphere”, “detritusphere”, “aging root” and “low response” (fig. 4) following definitions first described in [29]. Similarly, hierarchical clustering based on differential expression of the most highly upregulated *de novo* assembled ORFs compared to unamended bulk soil revealed clear rhizosphere and detritusphere guilds (sup. fig. S5). There was some overlap between guilds identified here and guilds identified based on expression of CAZy genes, but the degree of overlap varied by guild (sup. fig. S6).

Within the aging root guild, we noticed one member, representing an aggregated population transcriptome, that increased differential expression of extracellular proteases at the last time point in the presence of both roots and litter more than would have been predicted by the sum of the treatments alone. As this pattern differed from the rest of the guild, we identified this member as a sub-guild labeled “late synergist” due to its expression pattern. This MAG, Burkholderiales_62_29, was identified by two independent phylogenomic analyses as

Rhizobacter (sup. fig. S7). Burkholderiales_62_29 has 16 different extracellular protease genes, all of which had similar significant upregulation patterns. While the sub-guild here contained only a single *Rhizobacter* MAG, the implication of mapping transcriptomic reads at 80% identity is that each MAG represents a taxonomic "cloud" of at least genus-level diversity. A 16S-rRNA survey of the same samples revealed 10 operational taxonomic units (OTUs) of order Burkholderiales which, like *Rhizobacter*, are not assigned a family [29]. Additionally, this sub-guild may include more taxa for which we have no MAGs and therefore could not be included in this analysis.

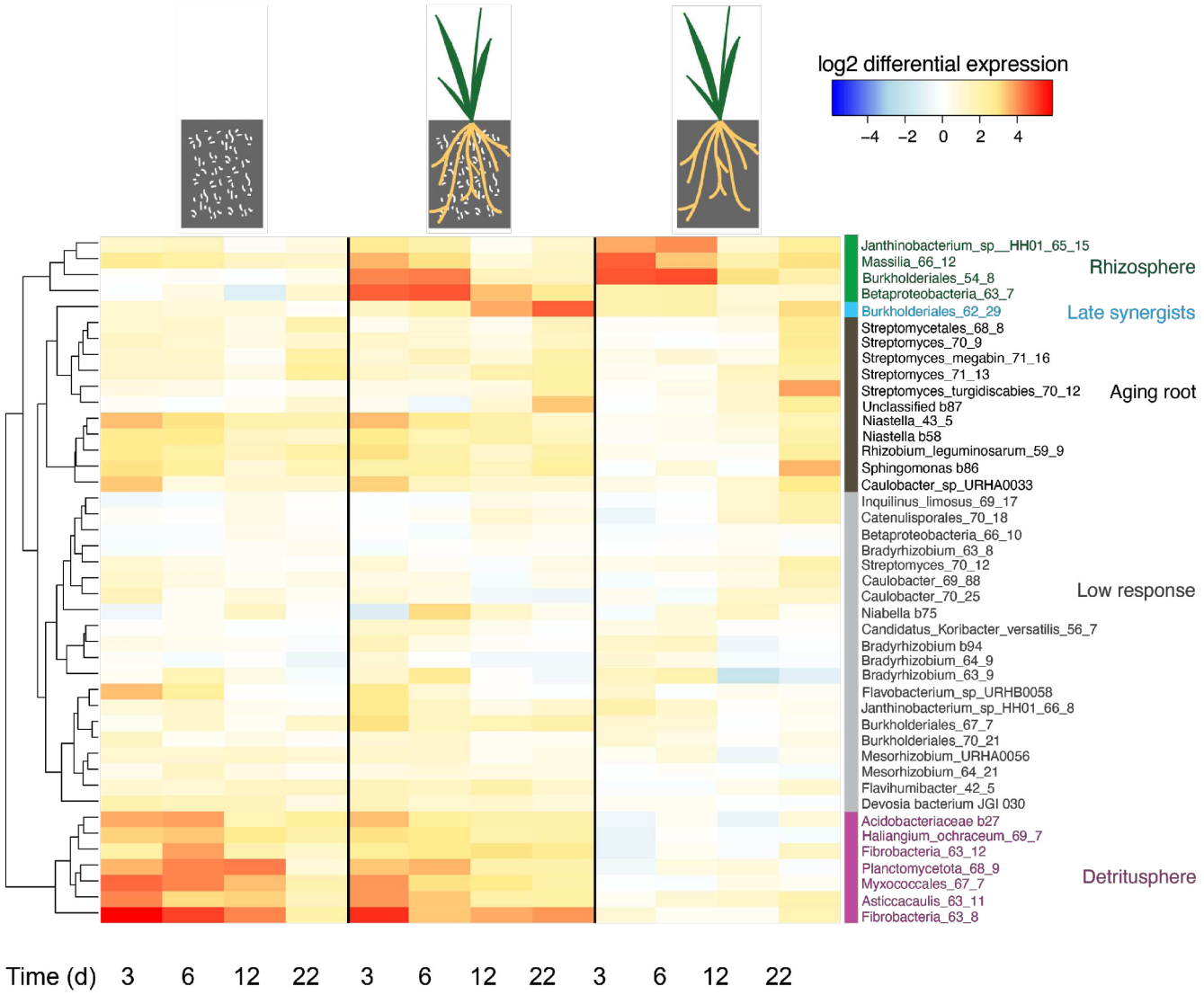

Figure 4: Functional guilds defined by hierarchical clustering of extracellular protease differential gene expression during a 22-day *Avena fatua* microcosm experiment. Each row represents the mean differential expression of extracellular protease genes mapped to a reference genome. Treatments are (left to right): litter-amended bulk soil, litter-amended rhizosphere and unamended rhizosphere. Time points are indicated at the bottom in days. We note that a genome may contain more than one exoprotease gene and that reads were mapped at 80% identity, therefore each genome also represents closely related taxa. Differential expression values per gene that were not statistically significant were converted to zero (0) before averaging.

Discussion

Nitrogen in grassland soil is present mostly in the form of organic macromolecules such as proteins and chitin [22,51]. Rhizosphere bacteria can acquire nitrogen through degradation of extracellular macromolecular N, and this process can be enhanced by root exudates especially in unfertilized or N limited soil [1,9,52]. Amino acids resulting from protein degradation are taken up by microorganisms, and roughly 30% of the amino-acid carbon is respired, leading to excretion of excess ammonium which could benefit the plant [53,54]. While various types of extracellular enzymes degrade macromolecules containing N, we focus here on protease and chitinase as they were significantly affected by litter amendment and by proximity to the plant (bulk soil vs. rhizosphere). A previous study based on the same experiment showed that there was very little change in the bacterial or fungal community composition throughout the experiment [29].

Chitinase expression, while two orders of magnitude lower than that of proteases, increased over time in litter-amended samples. This effect could imply that the litter attracted saprotrophic fungi and possibly arthropods, the cell walls of which contain chitin [55]. As most of the chitinases were most closely related to eukaryotic enzymes, it is likely that the chitin-containing hyphae were preyed upon by soil fauna. A previous study with *Avena fatua* in the same soil without a preceding addition of chitin showed that chitinase specific activity was highest near root hairs (several days old rhizosphere), medium in bulk soil and lowest near mature roots (one week old rhizosphere) [17]. In contrast, in rhizosphere with no litter addition, we saw little change in chitinase expression over time. The slight differences in these results may simply reflect the fact that the preceding study assayed enzyme activity and the current study assessed gene expression.

We found that in unamended soil, expression of extracellular proteases was higher in the rhizosphere compared to bulk soil, potentially resulting from input of carbon from root exudates creating a higher demand for nitrogen as well as microbial competition over inorganic nitrogen with the plant. Indeed, DeAngelis et al. showed that protease specific activity was significantly higher in young rhizosphere compared to bulk soil, but activity was not significantly different based on root age [17]. In litter-amended soil, expression of extracellular protease was highest regardless of location at 3 days, suggesting that litter-added carbon overwhelms the effect of root exudates at this early point in time. At 12 and 22 days there is no difference between litter-amended and unamended soil in either rhizosphere or bulk soil, implying that the effect of litter has waned. Changes in the quantity and quality of rhizodeposits can lead to changes in gene expression over time in the rhizosphere. An increased demand for N is consistent with the higher rates of gross nitrogen mineralization and ammonium consumption found in the rhizosphere of

393 mature compared to young *Avena sp*. [26]. The trend of increase in expression of extracellular
394 protease over time in our unamended rhizosphere soils suggests that as the rhizosphere ages it
395 becomes more nitrogen limited; thus, organic nitrogen would need to be mineralized to support
396 the microbial community.

397 Like many enzymatic reactions, macromolecular N depolymerization can be regulated by
398 substrate availability and end-product concentration [56]. In our study, litter amendment initially
399 led to high expression of extracellular proteases, suggesting that this expression was substrate
400 dependent [57,58] and linked carbon and nitrogen cycling [59]. However, protease activity in
401 grassland soil has also been shown to be affected by a multitude of parameters, such as end-
402 product concentration, aboveground net primary productivity [34] and soil aggregation [33]. We
403 observed an increase over time in expression in unamended samples although no nitrogen or
404 carbon sources were added. We hypothesize that this increase over time may be related to
405 depletion of end products (inorganic nitrogen), leading soil bacteria to invest more in expressing
406 depolymerization enzymes in order to access macromolecular nitrogen.

407 Spatiotemporal patterns of differential expression of genes were used to identify
408 functional guilds in which extracellular proteases were upregulated at a specific location or time.
409 These microbial guilds, which differed in taxonomic composition, underwent distinct
410 successional patterns that appear to be driven by niche conditions in the rhizosphere,
411 detritusphere, aging root and low response. Nuccio et al. [29], analyzing the same experimental
412 dataset, showed that microbial community composition (by 16S-rRNA) in each experimental
413 group changed very little compared to gene expression over the course of this experiment [29].
414 Therefore, the changing expression patterns over time that we observed are likely related to

changes in environmental conditions or cues, such as macromolecular N availability and inorganic N availability, as opposed to wholesale shifts in community composition.

Carbon and nitrogen cycling by soil bacteria are linked through substrates that contain both as well as by the need to maintain cellular C:N. The upregulation of carbon depolymerization may promote increased access to nitrogen through expression of extracellular proteases under the same conditions, leading to an overlap in guild membership. Such a causative correlation has previously been observed in loam agricultural soil using protease enzyme assays [59]. Therefore, we compared guild membership between guilds previously defined by CAZy expression [29] and guilds defined here by hierarchical clustering of extracellular protease expression. We found that rhizosphere guild membership was similar between CAZy and protease guilds, while the detritusphere guilds had little overlap. All members of the rhizosphere guild defined by extracellular proteases were also assigned to this guild by CAZy expression, and all were Betaproteobacteria known to be associated with rhizosphere soil [23,60,61]. In the sub-guild of "aging root" labeled "late synergist", Burkholderiales bin 62_29, was assigned to the rhizosphere guild by CAZy expression. The diverse detritusphere guild defined by extracellular protease expression included facultative predatory bacteria of the order Myxococcales (Deltaproteobacteria). Predatory bacteria produce proteases to bind to and digest prey bacteria [62] which may be attracted to litter. The small amount of overlap between the protease-defined and CAZy-defined detritusphere guilds could potentially be due to protein/amino acid supply of carbon backbones which reduces the need for degradation of carbohydrates by CAZy genes in extracellular protein degraders. Guild overlap or lack thereof contributes to further defining these multifunctional guilds in enzyme space.

Extracellular proteases have several structural types that differ in their active site. For example, metalloproteases require zinc in their active site, therefore their activity may by limited by zinc availability. There is currently very little information on when and where these different types are active in soil, but it has been suggested that their activity might depend on soil characteristics [32]. A recent publication by Nguyen et al. [14] showed that most of the extracellular proteases in soil and aquatic environments are Metallo-, Serine- and Cysteinepeptidases (MP, SP and CP respectively) in decreasing order of abundance. We found these same clusters were dominant in our data, but expression patterns from our study revealed dominance of SP over MP. Many bacterial phyla have dozens of copies of genes for secreted peptidase and these genes are enriched especially in Actinobacteria and Proteobacteria, and more so in soil/aquatic environments as opposed to animal associated bacteria. Specifically, Betaproteobacteria are enriched in SP (mean 13 SP copies out of a mean of 25 extracellular proteases per genome), with published genomes of isolates of Burkholderiales genera such as *Burkholderia*, *Janthinobacterium* and *Massilia* containing more than 50 and up to 155 copies per genome [14]. As Betaproteobacteria are enriched in the *Avena* rhizosphere [23], we might have expected higher expression of SP in this environment compared to bulk soil. Surprisingly, we did not observe that pattern; this could be explained by the high expression of genes from Actinobacteria in all groups, as they share the same enrichment pattern with Betaproteobacteria (SP>MP). Nguyen et al. also showed that while bacteria and archaea may carry genes coding for multiple types of these enzymes within the same genome, the enzyme profile of proteolytic activity was soil-specific [14,32]. Given that we see an expression profile of structural protease groups different from that expected from genomes, this may hint that soil bacteria modify expression of these groups in response to soil chemistry.

Conclusions

We show that extracellular proteases and chitinases are significantly upregulated by the presence of roots and/or plant litter compared to other macromolecular N degrading enzymes. Patterns of extracellular protease expression differ with taxonomy and can be used to define functional guilds. Three guilds, “rhizosphere”, “detritusphere” and “aging root” had membership overlap with guilds previously defined by carbohydrate degrading gene expression based on analysis of a CAZy genes dataset from the same experiment. This suggests that some of the members of these guilds co-express carbohydrate- and protein-degradation enzymes. These guilds also exhibited a pattern of temporal succession throughout plant maturation, where expression of protein degrading enzyme genes in the rhizosphere could be triggered by the host plant through control of exudation, or alternatively, may be a response to competition with the plant for labile nitrogen. The taxonomic resolution provided by metatranscriptomes opens a window to the identification of microbes that could enhance N availability to plants. The identity of these microbes has been previously obscured in bulk analyses such as enzyme assays, rate measurements and even qPCR. It is not far-fetched to imagine augmentation of soil or seeds with bacteria that specialize in macromolecular organic N degradation to reduce the use of fertilizers. Ideally, augmentation should use bacteria endemic to the specific plant rhizosphere. We show here that the identity of such bacteria depends on plant life stage and soil characteristics such as organic material load.

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Declarations

Ethics approval and consent to participate: not applicable

Consent for publication: not applicable

Competing interests: The authors declare that they have no competing interests

Availability of data and material: All data used in this publication, including raw reads and genome collection, has been published previously. Metagenomes assembled MAGs can be found in NCBI PRJNA517182, stable isotope probing MAGs in http://ggkbase.berkeley.edu/, single amplified genomes in IMG under study name Mediterranean Grassland Soil Metagenome and single amplified genomes in IMG, see sup. table S2 in Nuccio et al., 2020 for accession numbers [29]. Raw reads can be found in JGI IMG. For JGI ID's (accession numbers) see supplemental table S1 in Nuccio et al. 2020 [29].

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Authors' contributions: ETS performed the data analysis, data interpretation and drafted the manuscript. EEN, JPR and MKF conceived and designed the original rhizosphere-detritusphere study, EEN helped to interpret the data and revise the manuscript. JPR and MKF both interpreted the data and substantially revised the manuscript. All authors approved the submitted version and agreed both to be personally accountable for the author's own contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the

661 author was not personally involved, are appropriately investigated, resolved, and the resolution
662 documented in the literature.

Figures

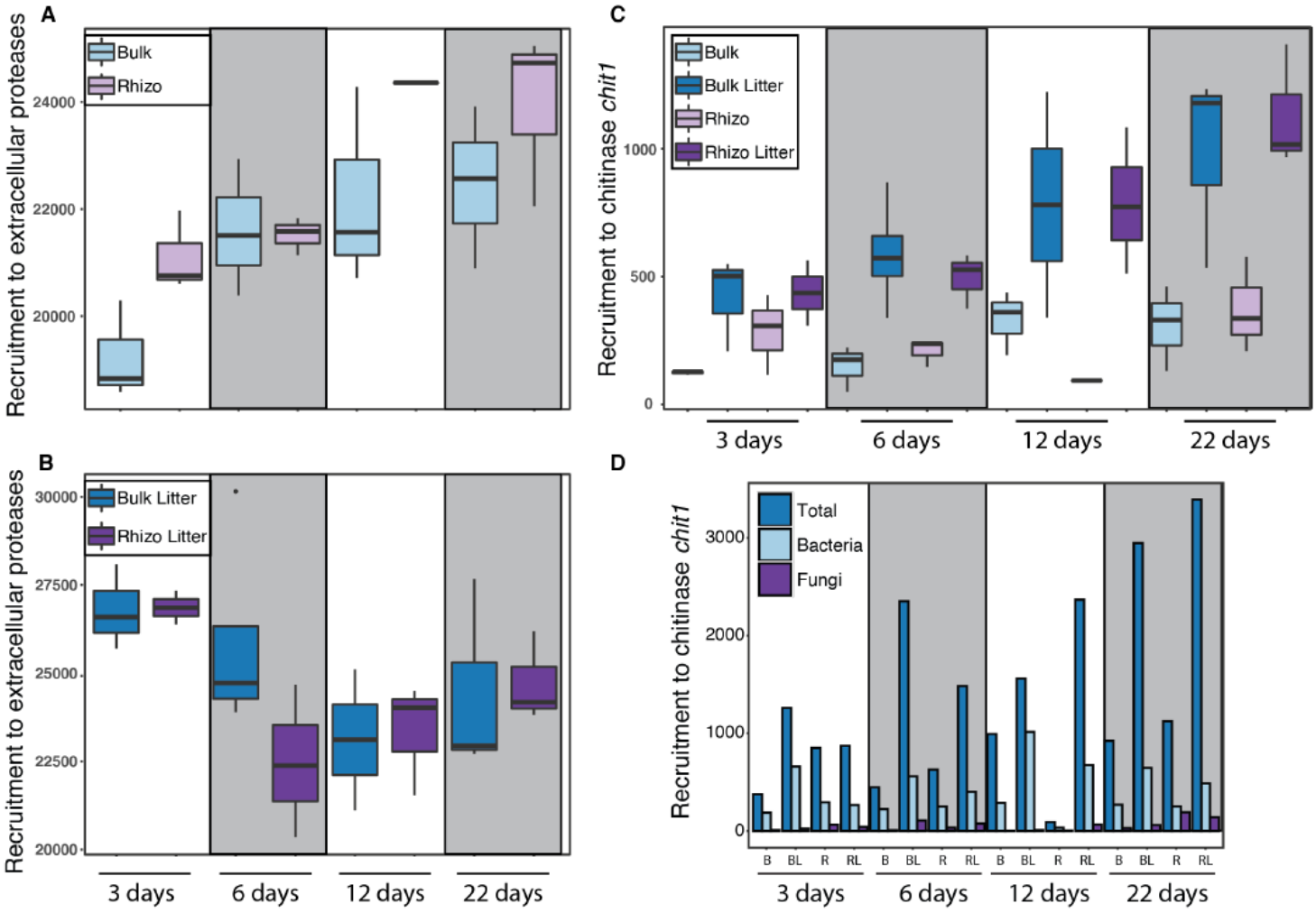

Figure 1

Aggregated expression of extracellular proteases in bulk and rhizosphere soils from common wild oat Avena fatua microcosms, grown without root litter amendment (A), or with litter amendment (B); expression of chitinase gene chit1 (C) and mean counts of chitinases at the domain level (D) for unamended bulk soil (B), litter-amended bulk soil (BL), unamended rhizosphere (R) and litter-amended rhizosphere (RL). Boxplots represent 75% of the data, whiskers denote 90% of the data and dots (e.g. panel A bulk litter 6 da ys) represent outliers. Note that the scale of the Y axis varies between panels.

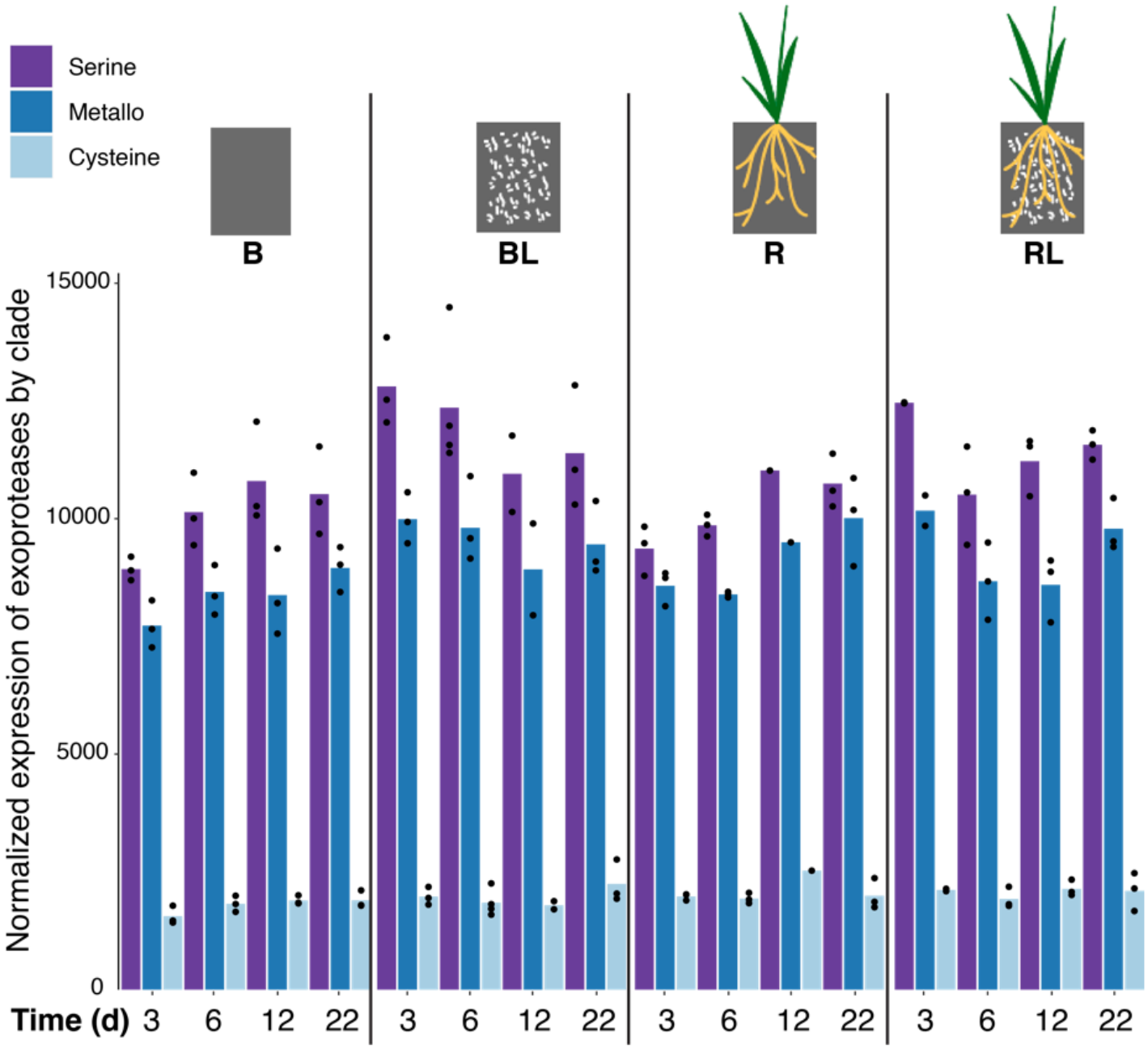

Figure 2

Aggregated normalized expression of extracellular protease structural groups over time in bulk, rhizosphere and root litter-amended soils. Normalized expression per replicate is plotted as dots on top of the bars. Experimental groups shown per time point are (left to right): unamended bulk soil (B), litter-amended bulk soil (BL), unamended rhizosphere (R) and litter amended rhizosphere (RL). Extracellular protease groups are (left to right): serine-, metallo- and cysteine-protease.

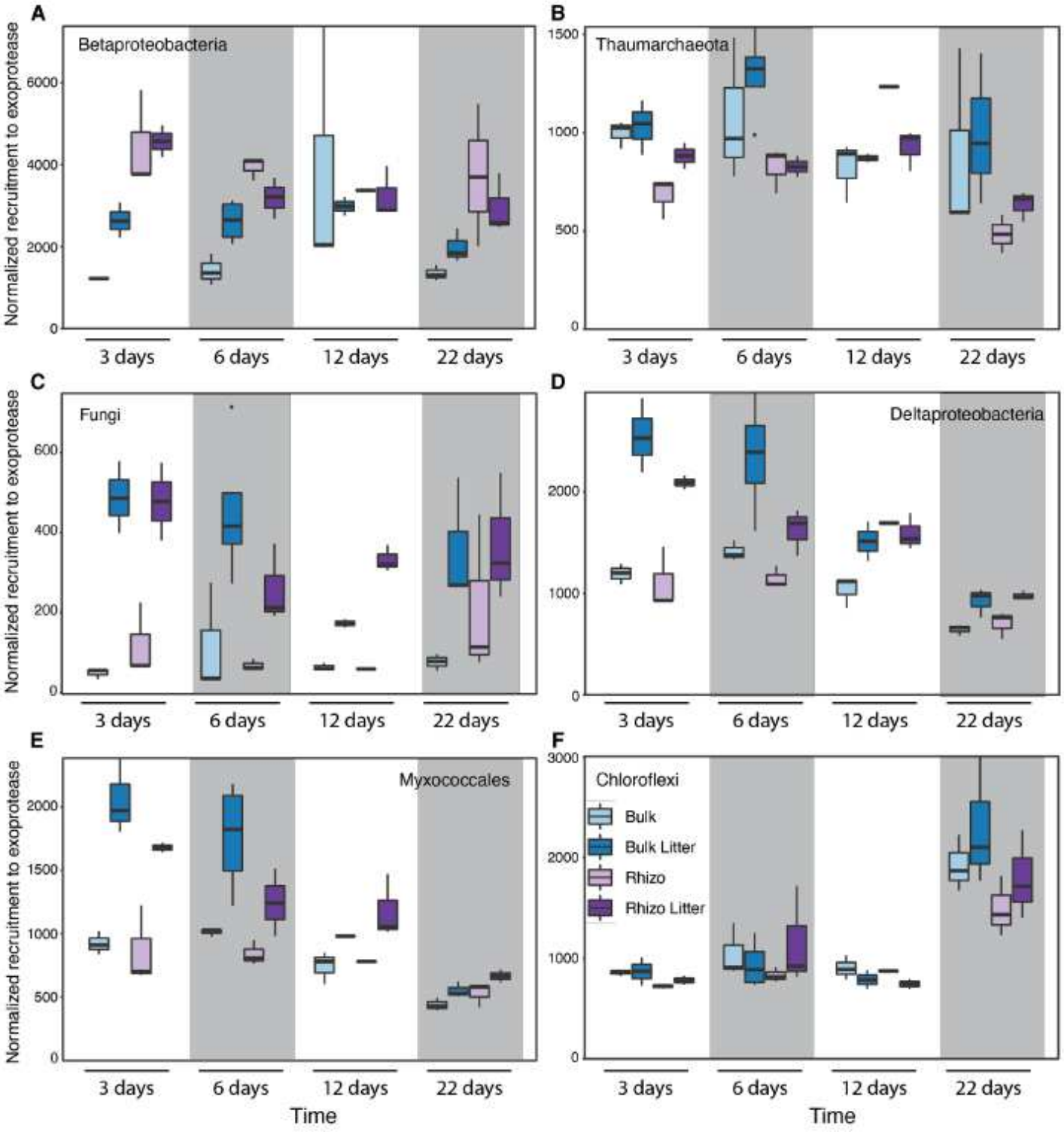

Figure 3

Expression of extracellular proteases over time for select taxonomic groups: (A) Betaprotebacteria (n=547) were upregulated in the rhizosphere, (B) Thaumarchaeota (n=112) were downregulated in the rhizosphere, (C) Fungi (n=99) were upregulated in the presence of litter, (D) Detaproteobacteria (n=228) were upregulated in the presence of litter, (E) Myxococcales (n=147) were upregulated in the presence of litter and decreased over time and (F) Chloroflexi (n=153) increased in the last time point. The legend in

panel F applies to all panels. Note that as expression levels varied over taxonomic groups and to emphasize patterns, the y axes do not have the same scale.

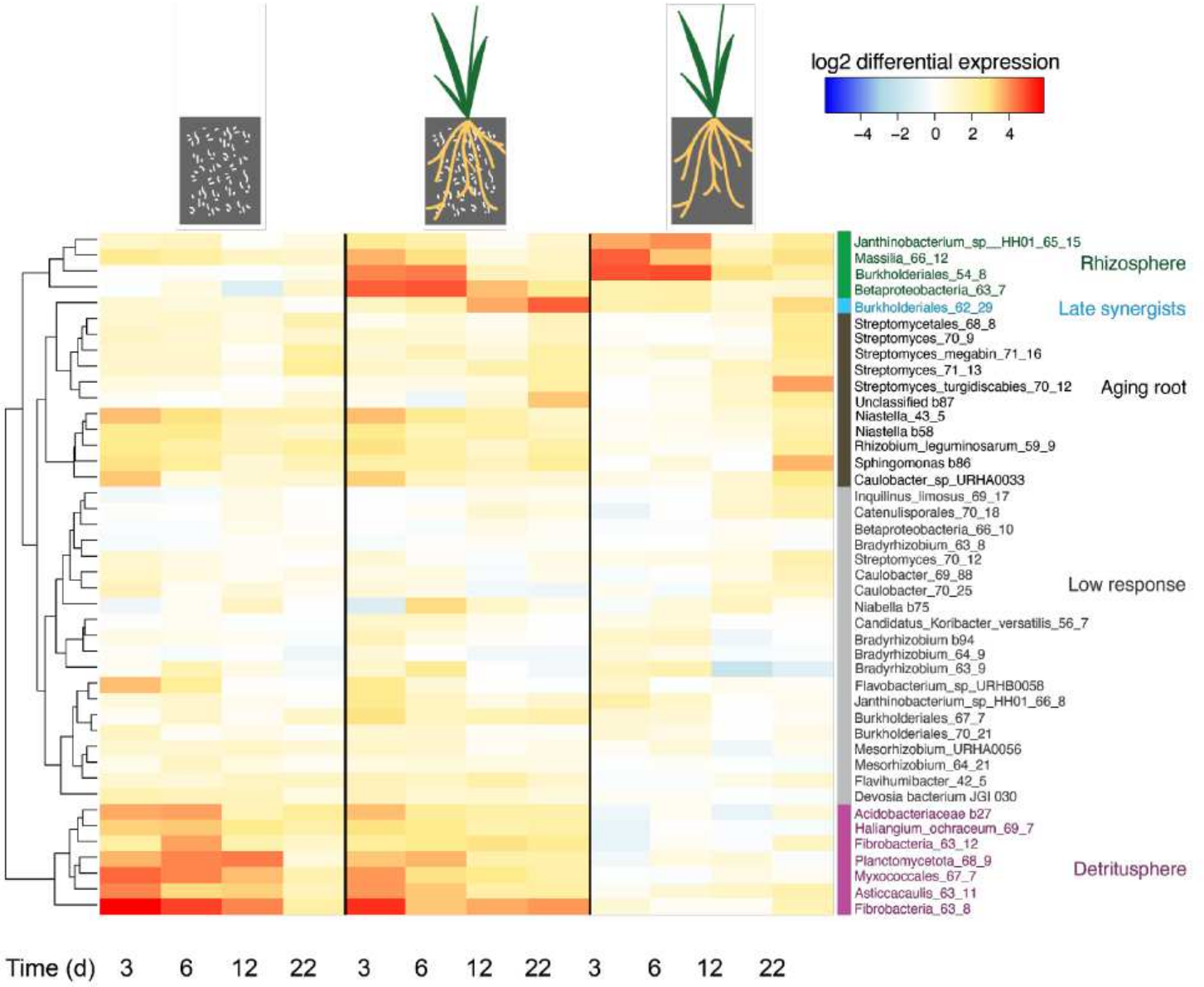

Figure 4

Functional guilds defined by hierarchical clustering of extracellular protease differential gene expression during a 22-day Avena fatua microcosm experiment. Each row represents the mean differential expression of extracellular protease genes mapped to a reference genome. Treatments are (left to right): litter-amended bulk soil, litter-amended rhizosphere and unamended rhizosphere. Time points are indicated at the bottom in days. We note that a genome may contain more than one exoprotease gene and that reads were mapped at 80% identity, therefore each genome also represents closely related taxa. Differential expression values per gene that were not statistically significant were converted to zero (0) before averaging.

Supplementary Files

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