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SHORT COMMUNICATION

Exploring the functional soil-microbe interface and exoenzymes through soil metaexoproteomics

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Functionally important proteins at the interface of cell and soil are of potentially low abundance when compared with commonly recovered intracellular proteins. A novel approach was developed and used to extract the metaexoproteome, the subset of proteins found outside the cell, in the context of a soil enriched with the nitrogen-containing recalcitrant polymer chitin. The majority of proteins recovered was of bacterial origin and localized to the outer membrane or extracellular milieu. A wide variety of transporter proteins were identified, particularly those associated with amino-acid and phosphate uptake. The metaexoproteome extract retained chitinolytic activity and we were successful in detecting *Nocardiopsis*-like chitinases that correlated with the glycoside hydrolase family 18 (GH18) *chi* gene data and metataxonomic analysis. *Nocardiopsis*-like chitinases appeared to be solely responsible for chitinolytic activity in soil. This is the first study to detect and sequence bacterial exoenzymes with proven activity in the soil enzyme pool.
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Metaproteomics is an emerging technique for directly assessing cellular function and interactions within an environment. In complex environments such as soil, there is a vast dynamic range of microbial species abundance and protein expression levels. Data acquisition is biased towards high-abundance proteins, for example, chaperonins, ribosomal proteins, elongation factors and ATP synthases (Benndorf *et al.*, 2007; Dill *et al.*, 2010). Removal of these intracellular proteins could allow access to functionally important low-abundance proteins in the soil enzyme pool and at the interface of cell and soil, the soil metaexoproteome.

Chitin provides one of the dominant sources of organic nitrogen in soil (Gooday, 1990) and chitinases are implicated in its mineralization in a wide range of contexts (Rhazi *et al.*, 2000; Muzzarelli, 2011), especially in nitrogen-poor soils (Olander and Vitousek, 2000). The molecular diversity of chitinases in soil microbial communities has been studied (Williamson *et al.*, 2000; Metcalfe *et al.*, 2002; Hjort *et al.*, 2010) but very few have focused on the functional contributions of members of the chitinolytic bacterial community. We report here the first attempt to recover and analyse extracellular proteins in soil adopting a novel approach to extract the metaexoproteome. Our data indicate that one actinobacterial group was disproportionately responsible for chitin breakdown.

Soil was sampled from an island off the north coast of Cuba known for its high biodiversity and wide range of chitinolytic bacteria (Williamson *et al.*, 2000; Williamson, 2001). Microcosms were constructed and amended with 1% crude crab shell (α-chitin) or squid pen (β-chitin) to enrich the microbial community, an unamended control was included for the 16S rRNA gene metataxonomic analysis (Supplementary Method S1). Community DNA was extracted and sequenced on a 454 GS FLX instrument with titanium reagents (Roche, Basel, Switzerland) using eubacterial primers Gray28F and Gray519R (Dowd *et al.*, 2008) and GH18 Group A *chi* primers GASQF and GASQR (Williamson *et al.*, 2000); the data were analysed with the bioinformatics package QIIME (Caporaso *et al.*, 2010) (Supplementary Method S2). The metaexoproteome extraction is a modification of Masciandaro *et al.* (2008). In brief, 100 g soil was gently agitated with a K_2SO_4-based extraction solution (1:3 w/v) then the solid fraction and cells removed by centrifugation and filter sterilization before dilution (3:1 v/v) with 18.2 MΩ cm water and dialysis overnight. The retentate was concentrated to a final volume of ~1 ml by ultrafiltration and using a centrifugal concentrator for direct loading onto a one-dimensional SDS–polyacrylamide gel electrophoresis gel. Gel-dependent nanoflow liquid chromatography-tandem MS (nanoLC-MS/MS) analysis was

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performed and the resultant Micromass peak list files interrogated with the NCBInr database using the MASCOT search engine (Matrix Science, London, UK). The full list of proteins was filtered to remove the few eukaryotic proteins and hits with <2 significant unique peptides (Supplementary Methods S3 and S4).

To successfully target the metaexoproteome, cell integrity must be maintained. Minimal cell lysis during the extraction was demonstrated experimentally by spiking soil with *Escherichia coli* overexpressing His-tagged phosphoribosyl isomerase A in the cytoplasm and attempting to detect the His-tag in the extract by western blot (Supplementary Method S5), as no protein was detected we believe the method did not lyse cells. The majority of 52 recovered proteins were Gram-negative in origin and attributed to the extracellular fraction or outer membrane (Supplementary Tables S1 and S2). Across both amendments, 73% of proteins were predicted to have a signal peptide (Nielsen *et al.*, 1997), 13% to have transmembrane helices (Sonnhammer *et al.*, 1998; Krogh *et al.*, 2001), 17% to be TRAP transporters and 52% to be ABC transporters. These features are suggestive of export or being membrane bound and indicate that the metaexoproteome is representing the functional interface between cell and environment. *In vitro* secretomes commonly feature a similar range of TRAP and ABC transporters in addition to selected extracellular enzymes depending on the enrichment (Adav *et al.*, 2010; Christie-Oleza and Armengaud, 2010; Christie-Oleza *et al.*, 2012). The only extracellular enzymes identified were chitinases.

Recovered proteins were affiliated with three phyla, *Proteobacteria*, *Actinobacteria* and *Bacteroidetes*, this correlated well with the 16S rRNA gene data set (Figure 1). Only two genera dominated the metaexoproteome, both in terms of number of proteins recovered and protein abundance measured by emPAI (Ishihama *et al.*, 2005), the actinomycete *Nocardiopsis* and the rhizobiale *Nitratireductor*. Approximately 17% of the identified proteins were matched to *Nocardiopsis*. The family *Nocardiopsaceae* was undetected in the unamended 16S rRNA gene data set but was one of the few actinobacterial groups to increase in abundance with α-chitin amendment, accounting for 3.7% of the community.

The majority of proteins were related to the transport and metabolism of amino acids, carbohydrates and inorganic ions, namely phosphate and phosphonate. Two GH18 chitinases were identified by peptides from within their catalytic domains, ChiA from *Nocardiopsis lucentensis* and *N. dassonvillei* (Supplementary Table S1). Corresponding *Nocardiopsis chiA*-like sequences were identified in the GH18 *chi* gene pyrosequencing data set (Figure 1). *Nocardiopsis* chitinases have been shown to have chitinolytic activity against α- and β-chitin (Tsujibo *et al.*, 2003) and to be capable of fast and complete degradation of crystalline chitin in liquid media (Sorokin *et al.*, 2012).

A fluorogenic chitinase assay (Sigma-Aldrich, St Louis, MO, USA) was performed on the extracts from α-chitin-amended microcosm soil and metaexoproteome (Supplementary Method S6). Both extracts showed activity against the monomeric substrate but the metaexoproteome extract had proportionally higher activity against the more representative di-NAG and tri-NAG substrates. It is probable that the chitinase activity detected in the metaexoproteome extract is attributable to the *Nocardiopsis chiA*-like chitinases detected in the sequenced aliquot of the extract and represents the first example of an active exoenzyme extracted, assayed and sequenced from a soil.

The efficiency of mass spectrometry via in-gel digestion would preclude recovery of low-abundance peptides. *Nocardiopsis*-like proteins

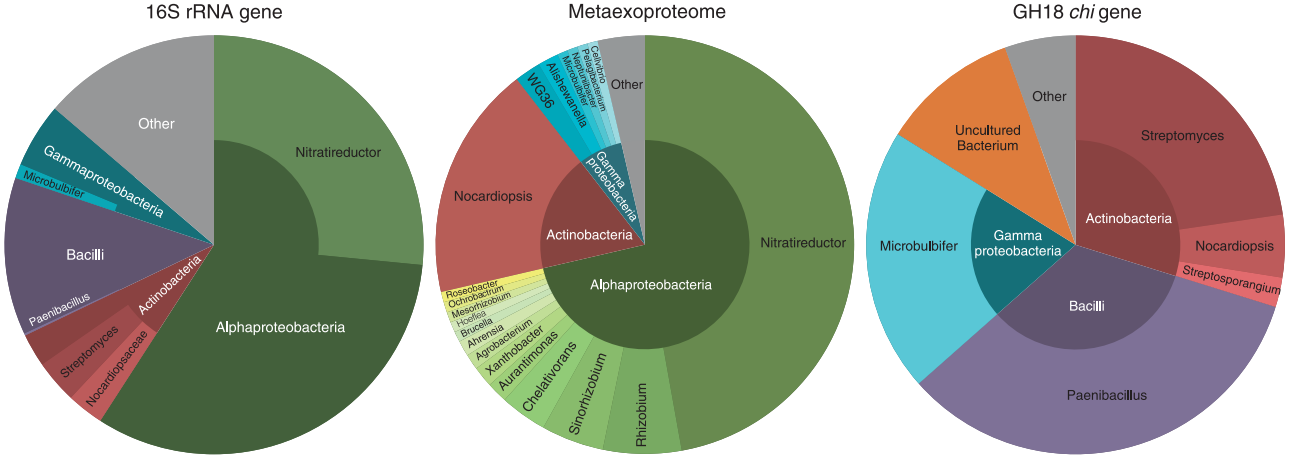

Figure 1 A visual summary of the assigned bacterial community structure, recovered metaexoproteome community and GH18 *chi* gene taxonomic matches for the combined α- and β-chitin-amended soil. For clarity, low-abundance taxa have been grouped under 'Other' and for the GH18 *chi* gene pie chart *Stenotrophomonas*, *Amycolatopsis* and *Verrucosispora* are not labelled as each account for <0.06% of their respective class segment.

must therefore contribute disproportionately to the functional activity of the soil and thus the degradation of chitin. This is in marked contrast to the prevalence data for 16S rRNA gene analysis and GH18 *chi* gene analysis. Despite numerous attempts it was not possible to cultivate *Nocardiopsis*-like strains directly from the soil.

Conflict of Interest

The authors declare no conflict of interest.

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