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Quantitative iTRAQ-based secretome analysis reveals species-specific and temporal shifts in carbon utilization strategies among manganese(II)-oxidizing Ascomycete fungi

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ABSTRACT

Fungi generate a wide range of extracellular hydrolytic and oxidative enzymes and reactive metabolites, collectively known as the secretome, that synergistically drive plant litter decomposition in the environment. While secretome studies of model organisms have greatly expanded our knowledge of these enzymes, few have extended secretome characterization to environmental isolates, particularly filamentous Ascomycetes, or directly compared temporal patterns of enzyme utilization among diverse species. Thus, the mechanisms of carbon (C) degradation by many ubiquitous soil fungi remain poorly understood. Here we use a combination of iTRAQ proteomics and extracellular enzyme activity assays to compare the protein composition of the secretomes of four manganese(II)-oxidizing Ascomycete fungi over a three-week time course. We demonstrate that the fungi exhibit striking differences in the regulation of extracellular lignocellulose-degrading enzymes among species and over time, revealing species-specific and temporal shifts in C utilization strategies as they degrade the same substrate. Specifically, our findings suggest that *Alternaria alternata* SRC1lrK2f and *Paraconiothyrium sporulosum* AP3s5-JAC2a employ sequential enzyme secretion patterns concomitant with decreasing resource availability. *Stagonospora* sp. SRC1lsM3a preferentially degrades proteinaceous substrate before switching to carbohydrates, and *Pyrenochaeta* sp. DS3sAY3a utilizes primarily peptidases to aggressively attack carbon sources in a concentrated burst. This work highlights the diversity of operative metabolic strategies among understudied yet ubiquitous cellulose-degrading Ascomycetes, enhancing our understanding of their contribution to C turnover in the environment.

1. Introduction

Fungal secretomes contain a diverse suite of extracellular enzymes and reactive metabolites that facilitate the breakdown of recalcitrant plant and animal material in the environment. Specifically, fungi secrete an arsenal of diverse hydrolytic and oxidative enzymes, including cellulases, hemicellulases, pectinases, and lignin-degrading accessory enzymes that generate reactive oxygen species (ROS), which synergistically drive litter decomposition in natural systems and can be harnessed for industrial applications (Hatakka and Hammel, 2010; Dashtban et al., 2010; Ruiz-Duenas and Martinez, 2009; Perez et al., 2002). As such, fungal secretomes play a critical role in global carbon cycling and climate dynamics and serve as an essential mediator in renewable energy production.

The ongoing development of analytical techniques in microbial 'omics has allowed researchers to delve more deeply into the mechanistic underpinnings of complex microbially-mediated processes in the environment, generating large, highly informative datasets that shed

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new light on the intricacies of microbial metabolism. In particular, comparative proteomics has proven to be a valuable tool in investigating the response of fungal secretomes to different growth conditions and environmental stimuli and evaluating these responses over time. By identifying enzymatic targets for future biochemical investigation and describing the dynamic regulatory profile of these secreted enzymes, researchers can begin to tease apart the diverse and elaborate mechanisms by which these organisms attack recalcitrant material, screen diverse organisms for hyper-production of enzymes of interest, and optimize the growth conditions and timing to yield a productive secretome harvest for industrial or commercial use.

Proteomic studies of the fungal secretome have already yielded interesting and informative results. By directly comparing the secretome composition of model organisms growing on different carbon sources, researchers have demonstrated that the suite of extracellular enzymes secreted by fungi is highly dependent on substrate composition, and that secretome size and functional diversity increase when the fungi are presented with more complex and recalcitrant material (Phalip et al., 2005; Medina et al., 2004; Lu et al., 2010; Liu et al., 2013; Girard et al., 2013). Taking these comparative analyses a step further, quantitative time-course investigations have illustrated dynamic shifts in metabolic strategies as fungi accumulate compounds of interest (Shi et al., 2013), adjust to diminishing resource availability (Nitsche et al., 2012), or alter enzyme regulation patterns as they sequentially degrade a recalcitrant substrate (Saykhedkar et al., 2012). In particular, these studies have provided valuable insights into lignocellulose degradation mechanisms by wood-rot Basidiomycetes, such as the secretion of species-specific suites of enzymes that are either diverse and synergistically acting or much more narrow in focus (Vanden Wymelenberg et al., 2010), and predictable patterns of sequential enzyme secretion that transition from specific to non-specific oxidative mechanisms over time as the remaining substrate becomes more difficult to degrade (Hori et al., 2014). As more data emerge, patterns common to diverse fungi are elucidated, such as the combination of glucanases, cellobiose dehydrogenase, and lytic polysaccharide monooxygenases that work together to accelerate lignocellulose degradation after the breakdown of more easily accessible compounds (Saykhedkar et al., 2012; Hori et al., 2014; Langston et al., 2011).

While our knowledge surrounding the extracellular enzymes secreted by filamentous fungi is expanding rapidly, few studies have extended secretome characterization efforts beyond model organisms (such as Ascomycetes in the *Aspergillus* (Lu et al., 2010; Liu et al., 2013) and *Fusarium* (Phalip et al., 2005) genera and white-rot Basidiomycetes (Vanden Wymelenberg et al., 2010; Rohr et al., 2013) to environmental isolates. As such, the mechanisms underlying their contribution to recalcitrant carbon degradation in terrestrial systems remain poorly understood. Furthermore, due to the inherent complexity in mapping proteins across multiple genomes, particularly those of phylogenetically diverse taxa, side-by-side comparisons of multiple species have thus far been limited (see Shi et al., 2013 for an example using yeasts and Vanden Wymelenberg et al., 2010 for wood decay Basidiomycetes). However, preliminary work on interspecies comparisons has resulted in valuable mechanistic insights and warrants additional study and extension of this work to a broad range of environmental isolates. In particular, lignocellulose-degrading filamentous Ascomycetes are ideal candidates for such investigations, since genomic and proteomic information on these Ascomycetes currently lags behind more well-studied Basidiomycete fungi, and thus their carbon cycling mechanisms remain enigmatic.

We have recently isolated over a dozen strains of manganese (Mn) (II)-oxidizing, filamentous Ascomycetes from metal-contaminated field sites (Santelli et al., 2010, 2014). Here we focus on four of these strains: *Alternaria alternata* SRC1lrK2f, *Paraconiothyrium sporulosum* AP3s5-JAC2a, *Pyrenochaeta* sp. DS3sAY3a, and *Stagonospora* sp. SRC1lsM3a. These fungi exhibit varied lifestyles (e.g., saprotrophic and plant pathogenic), are present in soil ecosystems worldwide, and have recently-sequenced genomes to facilitate 'omics-based investigations (Zeiner et al., 2016). Although they have demonstrated cellulose-degrading capacity (C.M. Santelli, unpublished data), the mechanisms underlying this process are unknown. Furthermore, it is unclear whether the ability of these four fungi to oxidize Mn(II) is linked to their ability to break down cellulose and potentially lignin, as it is in model white-rot Basidiomycetes such as *Phanerochaete chyrsosporium* (Höfer and Schlosser, 1999; Glenn et al., 1986; Wariishi et al., 1992; Schlosser and Höfer, 2002).

We have previously demonstrated that the secretomes of the above-mentioned four fungal species exhibit a rich functional diversity of proteins, suggesting that their cellulose-degrading capacity may involve both direct enzymatic carbohydrate breakdown as well as indirect carbon oxidation via Fenton-based hydroxyl radical formation (Zeiner et al., 2016). In this study, we extend this work to identify the dynamic enzyme regulation patterns among species in batch culture over a three-week time course. Utilizing iTRAQ (isobaric tags for relative and absolute quantification) proteomics, a custom bioinformatic pipeline, and extracellular enzyme activity assays, we reveal species-specific and temporal shifts in carbon utilization strategies among the four fungi as they degrade the same substrate under the same growth conditions. This work highlights the utility of iTRAQ proteomics in evaluating interspecies comparisons, demonstrates the diversity of operative metabolic strategies among cellulose-degrading filamentous Ascomycetes, and enhances our understanding of the role of these fungi in recalcitrant carbon turnover in the environment.

2. Materials and methods

2.1. Fungal species and culture medium

We investigated four filamentous Ascomycete fungi isolated from two locations. Three species were isolated from passive coal mine drainage treatment systems in central Pennsylvania that remediate high concentrations of Mn (Santelli et al., 2010): *Alternaria alternata* SRC1lrK2f, *Stagonospora* sp. SRC1lsM3a, and *Pyrenochaeta* sp. DS3sAY3a. The fourth species was isolated from Ashumet Pond, Massachusetts, a natural freshwater lake (Santelli et al., 2014): *Paraconiothyrium sporulosum* AP3s5-JAC2a. This field site was historically polluted with elevated concentrations of phosphate and metals, including Fe and Mn, and is currently undergoing remediation. All four fungi are classified in the *Pezizomycotina* sub-phylum.

All fungal species were grown in HEPES-buffered (20 mM, pH 7) acetate-yeast extract (AY) + Mn medium, which contains 0.25 g L^{-1} sodium acetate, 0.15 g L^{-1} yeast extract, and 1 mL L^{-1} trace element stock (10 mg L^{-1} $CuSO_4{\cdot}5H_2O$, 44 mg L^{-1} $ZnSO_4{\cdot}7H_2O$, 20 mg L^{-1} $CoCl_2{\cdot}6H_2O$, and 13 mg L^{-1} $Na_2MoO_4{\cdot}2H_2O$) supplemented with $MnCl_2$ (200 μM). All chemicals were reagent grader or higher. Fungal cultures were maintained on petri dishes of agar-solidified (2% agar) AY + Mn.

2.2. Culture conditions and secretome harvesting

Liquid cultures consisting of 100 mL AY + Mn were inoculated with homogenized inocula of each of the 4 fungi as previously described (Zeiner et al., 2016) and incubated at room temperature, without agitation, for 7, 14, or 21 days. For each fungus at each time point, five individual 100 mL cultures were combined into a 500 mL sample. Each 500 mL sample was considered one biological replicate. In total, forty-eight 500 mL samples were prepared for secretome characterization (4 fungi × 3 time points × 4 biological replicates; a total of 240,100 mL cultures). For each biological replicate, five 100 mL cultures were generated instead of one large 500 mL culture to increase biomass generation and decrease incubation times. Individual 100 mL cultures were assigned to each biological replicate at the time of inoculation to eliminate bias when combining 100 mL cultures into 500 mL samples.

Upon harvesting the samples, bulk biomass was removed and discarded, and the spent medium was filtered through a 0.45 μm