SAND2019-4692C

Transport of lignin-breakdown products into bacteria and fungi

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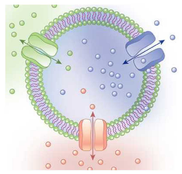
Abstract: Lignin is typically 15-28% of the total dry weight of biomass, making it a significant potential source of revenue, yet it is currently a waste stream in biofuel production that is usually burned to generate power. Efficient, controllable conversion of lignin to useful molecular building blocks has been elusive. While much lignin breakdown occurs extracellularly, the breakdown products, a substantial fraction of which are mono- and di- aromatics, are known to be taken up by lignolytic fungi and bacteria and metabolized intracellularly.[1-5] Assimilation of these breakdown products is an important part of lignin conversion, yet very little is currently known about the transporters that shuttle these compounds in lignolytic organisms.[6,7] Additionally, the substrate range and kinetics of transport into microbes are also not known. We performed an initial study of native transport of 4-hydroxybenzoic acid (HBA), vanillic acid (VA), *p*-coumaric acid (CA), syringic acid (SA) and the model dimer guaiacylglycerol beta-guaiacyl ether (GGE) into *Enterobacter lignolyticus*, *Escherichia coli, Pseudomonas putida*, *Phanerochaete chrysosporium,* and *Saccharomyces cerevisiae*. Internalization and accumulation of each compound was determined via LC-MS of the cell lysate. Additionally, click-chemistry-enabled fluorescence microscopy was performed for HBA, VA and GGE analogues to determine single cell uptake efficiency. For *Pseudomonas putida* the monomeric compounds were internalized and subsequently metabolized whereas GGE was internalized but was not metabolized. Other notable results include very high accumulation of vanillic acid in *Escherichia coli* and of coumaric acid in *Saccharomyces cerevisiae*. This work shows that uptake of lignin breakdown products can be enhanced through expression of exogenous transporters.

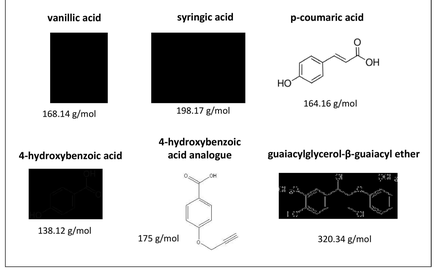
Experimental Overview



Left: Illustration of transport of lignin breakdown products into bacteria or fungi. © 2010 Nature Education

Below: Diagram of mono- and di-aryl compounds tested.

Compounds tested to date:



Organism and Growth Conditions:

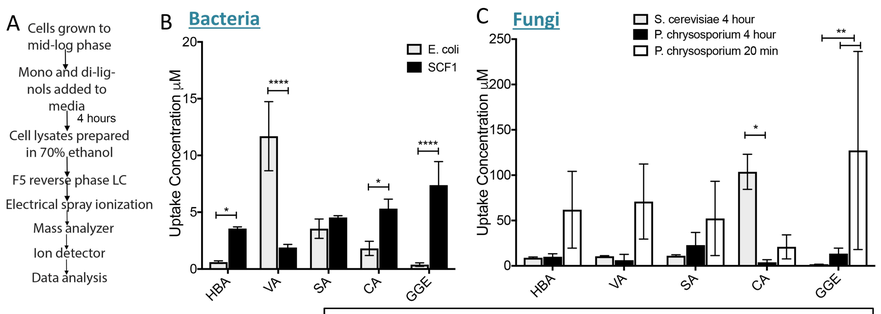
***P. chrysosporium* (IFO 31249, ATCC 34541)**: Grown at T=37 °C in Kirk's mineral salts and trace elements, 2 mg/ml microcrystalline cellulose, 20 mM ammonium tartrate. Internalization assays done on day 5 after innoculation. Fungal media was replaced with media or PBS containing 4 mM of target compound. After a 20 min,. 4 or 72 hr incubation period, the contents of culture were collected, the pellet was washed 2 times with PBS, then vortexed for 2 hours with 70% EtOH and lysing matrix C, and filtered through 3KDa cutoff filter. Filtrate was analyzed by LC/MS.

***S. cerevisiae* (BY4741, ATCC 201388)**: The trial was initiated after cultures reached an OD of 1.0, grown at 30 °C. 4mM compound stock solution in the media (YPD) were added to each culture. Cells were grown for 4 hours then harvested, washed, lysed, filtered, and analyzed by LC/MS protocol.

E. lignolyticus SCF1, E. coli (MG1655),* and *P. putida (KT2440): Cells were grown to mid log phase in minimal M9 media + 0.4% glucose (*E-coli, P. putida*) or rich media (*E. ligno*) and then compound stock solution in the media was added to 4 mM and allowed 4 hours for uptake. Harvesting, lysing, filtration, and MS protocols were the same.

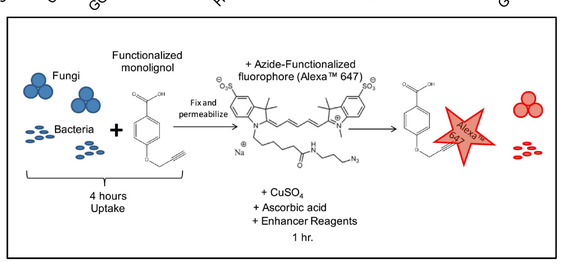
Results:

Internalization of Mono- and Di-arvls bv LC/MS

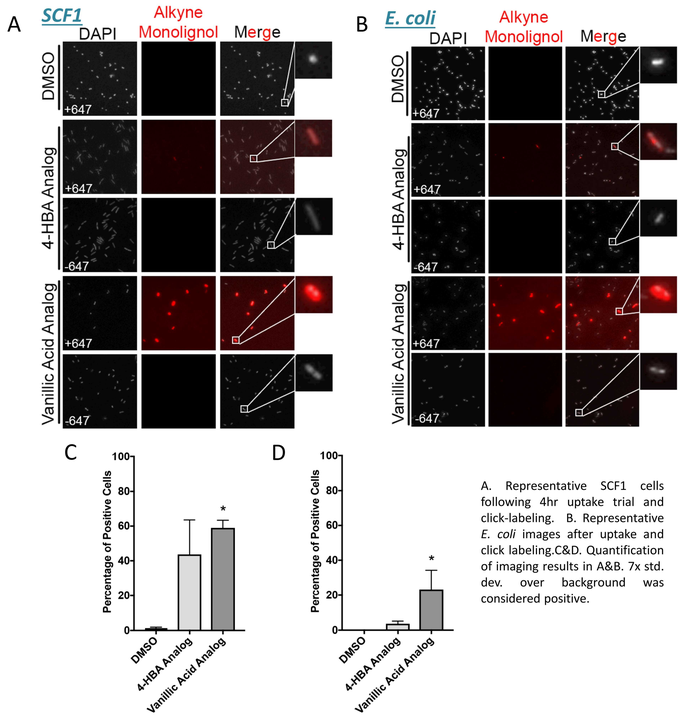


Above: A. LC-MS Workflow B. Relative concentration (uM) of each compound in lignolytic (*SCF1*) vs. non-lignolytic bacteria (*E. coli*) following 4 hours of internalization. C. Relative concentration (uM) of each compound in lignolytic (*P. chrys.*) vs. non-lignolytic fungi (*S. cere.*) following 4 hours of internalization. Relative concentrations determined using standards spiked into lysate at 5 µM.

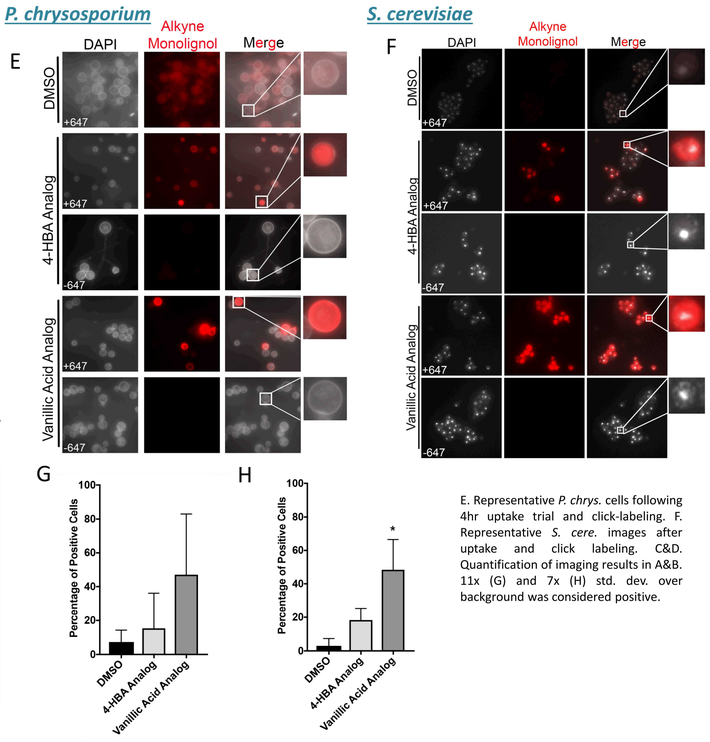
Right: 4-HBA and vanillic acid alkyne schematic for Click-chemistry labeling



Internalization of 4-HBA and VA Analogues in Bacteria and Fungi by Single Cell Microscopy

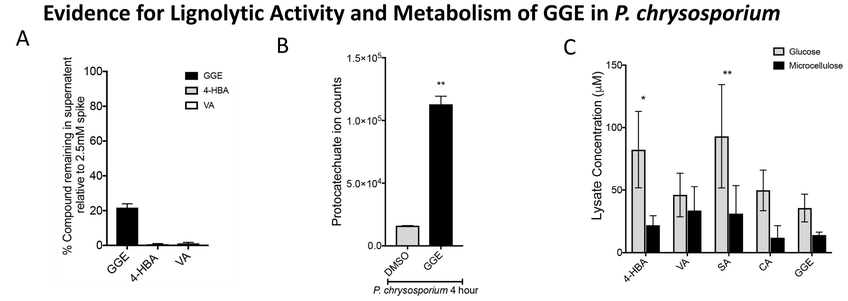


A. Representative SCF1 cells following 4hr uptake trial and click-labeling. B. Representative *E. coli* images after uptake and click labeling.C&D. Quantification of imaging results in A&B. 7x std. dev. over background was considered positive.



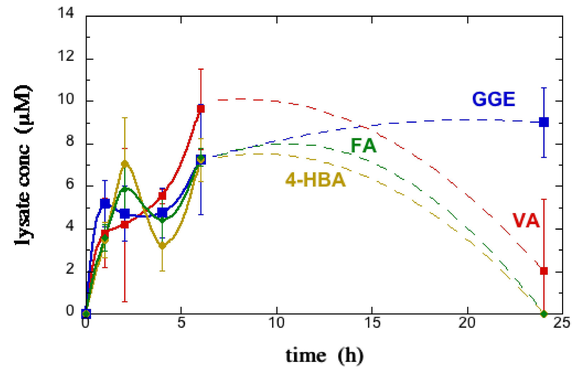
E. Representative *P. chrys.* cells following 4hr uptake trial and click-labeling. F. Representative *S. cere.* images after uptake and click labeling. C&D. Quantification of imaging results in A&B. 11x (G) and 7x (H) std. dev. over background was considered positive.

Evidence for Lignolytic Activity and Metabolism of GGE in *P. chrysosporium*



A. HPLC results of indicated compounds remaining in media after 72 hours internalization by *P. chrys*. grown under minimal conditions, indicating almost complete depletion of the mono- and di- aryls. B. LC/MS average ion counts for protocatechuate (GGE metabolite) in DMSO and GGE-exposed *P. chrys.*, C. LC/MS results of 20 minute *P. chrys.* uptake trial in media containing glucose or microcellulose (MC). MC induces lignolytic pathways in *P. chrys* and upregulates metabolism of the breakdown products. Surprisingly, uptake levels were comparable in lignolytic and nonlignolytic conditions.

Time dependent internal concentrations of VA, FA, 4-HBA, and GGE for *P. putida*



The results how that all compounds are taken up by *P. putida*. The intracellular concentrations of the monomeric compounds VA, FA, and 4-HBA decrease between 8 h and 24 h whereas the intracellular concentration of GGE increases over that time period. The monomeric compounds are either metabolized by *P. putida* or are effluxed, whereas GGE is not metabolized or effluxed to the same extent as the monomeric compounds.

References

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Conclusions

- LC-MS and Click-chemistry-mediated fluorescence imaging methods developed for measuring uptake
- Non-lignolytic organisms – *E. coli* and *S. cerevisiae* – have basal levels of transport for these compounds, except:
  - High uptake of vanillic acid by *E coli*, high uptake of coumaric acid by *S cerevisiae*
- Lignolytic organisms generally display enhanced internalization relative to non-lignolytic hosts (esp. true for GGE)
- *P. chrys* shows greater internal levels of aryls at 20 min than at 4 hr, apparently due to metabolism.
  - Identification of metabolites and depletion of compounds from supernatant further support metabolism.
- *P. putida* internalizes and metabolizes or effluxes the monomeric compounds VA, FA, and 4-HBA, whereas *P. putida* internalizes GGE but does not metabolize or efflux it to the same extent.

Funding and Acknowledgements

This work was supported by the Laboratory Directed Research and Development Program at Sandia National Laboratories.

Sandia National Laboratories is a multi-mission laboratory managed and operated by National Technology and Engineering Solutions of Sandia, LLC., a wholly owned subsidiary of Honeywell International, Inc., for the U.S. Department of Energy's National Nuclear Security Administration under contract DE-NA-0003525. We acknowledge the DeAngelis group at the University of Massachusetts Amherst for providing us with the SCF1 strain, and Christina Beppler and Kristin Cochran (SNL) for mass spectrometry analysis.