Exoproteomics reveals outer membrane vesicle mediated catabolism of lignin-derived aromatics by *P. putida* KT2440

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Abstract

Lignin is an abundant component of plant cell walls which is heterogeneous and highly resistant to degradation. Biological funneling of lignin via microbes represents an efficient approach for reducing chemical heterogeneity and generating molecules which can be valorized into performance-advantaged materials. *Pseudomonas putida* KT2440 (*P. putida*) catabolizes lignin-derived aromatic monomers rapidly and efficiently via the ß-ketoadipate (ßKA) pathway and our data suggest *P. putida* may depolymerize HMW lignin as well [1]. The mechanism by which HMW lignin is depolymerized by bacteria remains unknown. Intriguingly, we found that *P. putida* secretes outer membrane vesicles (OMVs) of two distinct sizes in the presence of lignin. Here, we describe the temporal proteome of OMVs and the vesicle-free secretome (VFS) in the presence or absence of lignin. Enzymes involved in aromatic catabolism via the ßKA pathway are temporally enriched into OMVs in the presence of lignin. Isolated OMVs enhance growth of *P. putida* mutants unable to grow on model lignin-derived aromatics, and turnover protocatechuic acid *in vitro*. Together, this work leads us to hypothesize mechanisms by which OMVs contribute to extracellular aromatic catabolism by *P. putida*. Ongoing and future work seeks to further understand this phenomena in the context of both fundamental and applied lignin conversion research areas.

P. putida can be used to valorize lignin—a recalcitrant polymer in biomass—into performance-advantaged products

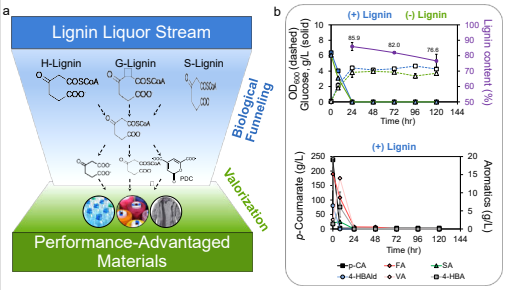

Fig. 1. (a) Corn stover can be alkaline pretreated to generate lignin-rich liquor streams and polysaccharide-rich solids. The resulting heterogeneous and recalcitrant lignin liquor can be fed to bacteria, such as *P. putida*, for "biological funneling" via the ß-ketoadipate pathway. Metabolic engineering approaches enable **biological production of industrially useful molecules from lignin** with great selectivity. Polymerization of these molecules into performance-advantaged materials, such as plastics, nylons, and textiles, can then be accomplished chemically. (b) Growth of *P. putida* on minimal media with and without lignin, showing **rapid & complete utilization of lignin-derived monomers and slow utilization of HMW lignin by *P. putida* KT2440.**

P. putida secretes outer membrane vesicles of distinct sizes on lignin

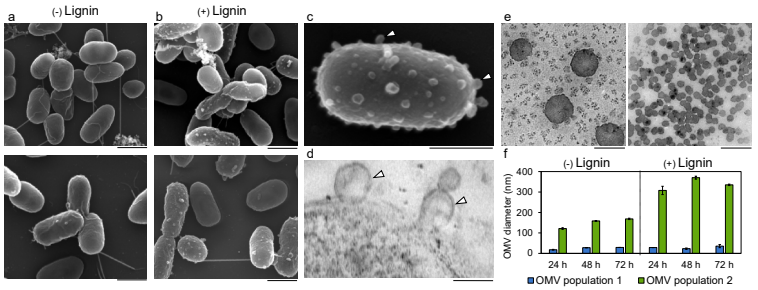

Fig. 2. SEM images of P. putida cultivations after three days on minimal media (a) without lignin and (b) with lignin. Scale bar = 1 µm. (c) SEM image of P. putida cell with OMV blebbing events marked with white arrow head. Scale bar = 0.5 µm. (d) TEM image of an OMV blebbing event from the outer membrane (*) of an embedded and sectioned P. putida cell. Scale bar = 0.1 µm. OMV blebbing events marked with white arrow heads. Analysis of the two distinct sizes of OMVs observed in P. putida cultivations on lignin after three days of growth by (e) TEM and (f) DLS. (e) Scale bars = 0.5 µm. (e) Error bars represent the standard deviation across biological triplicates with technical replicates.

The OMV proteome is dynamic across cultivation time and responsive to lignin

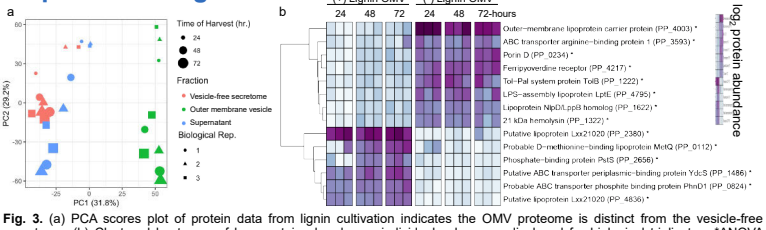

Fig. 3. (a) PCA scores plot of protein data from lignin cultivation indicates the OMV proteome is distinct from the vesicle-free secretome. (b) Clustered heat map of $\log_2$ protein abundance; individual values are displayed for biological triplicates. *ANOVA Tukey's HSD, p<0.05.

OMVs enhance catabolism of lignin-derived aromatics via the ß-ketoadipate pathway

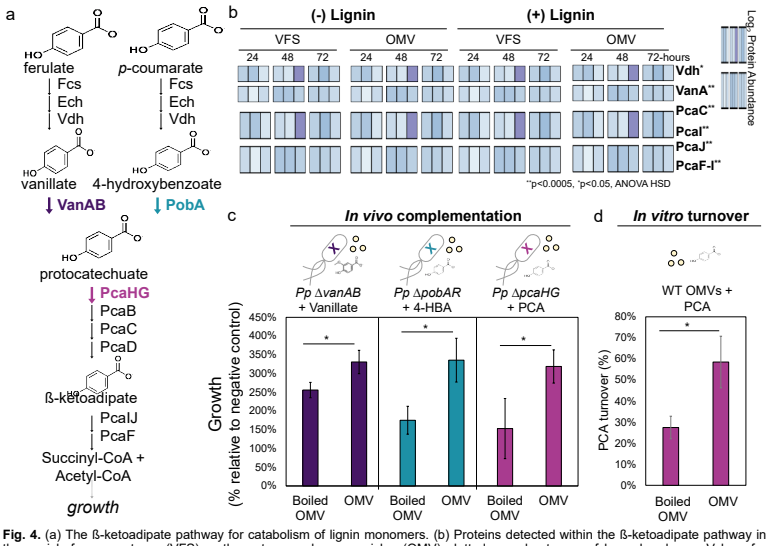

Fig. 4. (a) The ß-ketoadipate pathway for catabolism of lignin monomers. (b) Proteins detected within the ß-ketoadipate pathway in the vesicle-free secretome (VFS) or the outer membrane vesicles (OMV) plotted as a heat map of $\log_2$ abundance. Values for biological triplicate at each time point are plotted individually. *p<0.05 and **p<0.005, ANOVA with Tukey's HSD of the VFS vs. OMV. (c) *In vivo* complementation after 72 hours displayed as the percent growth increase as compared to the knock-out strain which cannot grow on the provided aromatic alone. *p<0.05, pairwise *t*-test of the boiled OMV *vs.* OMV. (d) *In vitro* turnover of protocatechuate by enriched OMVs after 24-hours of incubation. *p<0.05, pairwise *t*-test of the boiled OMV *vs.* OMV.

Working hypotheses for OMV-mediated aromatic catabolism

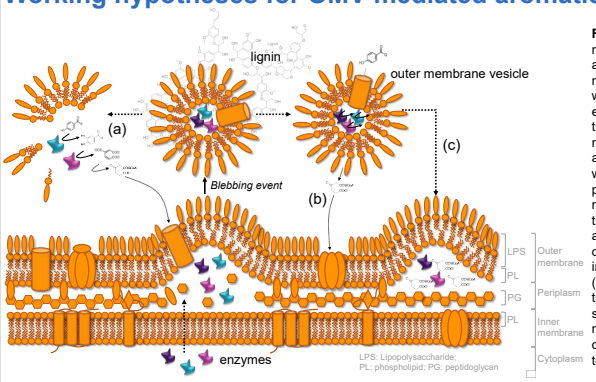

Fig. 5. Three models for OMV-mediated aromatic catabolism are presented. (a) OMV-mediated protein export whereby concentrated enzymes catalyze aromatic turnover in the extracellular milieu. (b) OMV export and aromatic turnover *intra*-OMV, whereby enzyme function is protected from the extracellular milieu. Transport in and out of the OMV would be required and ring-cleaved products diffuse toward the cell for import and further catabolism. (c) OMV export and *intra*-OMV turnover as in (b), but with subsequent fusion with the cell membrane for concentrated delivery of OMV cargo directly to the cell.

Conclusions, future directions, & acknowledgements

P. putida secretes outer membrane vesicles concurrent with catabolism of lignin-derived aromatic compounds. The OMVs are stocked with enzymes involved in that catabolism via the ß-ketoadipate pathway. Isolated OMVs complement *in vivo* growth in *P. putida* strains which have been genetically modified to be unable to growth on aromatic compounds alone. Further, OMVs turnover protocatechuate—the central intermediate of the ß-ketoadipate pathway—*in vitro*. Together this data lead us to present working hypotheses for how OMV-mediated catabolism of lignin and/or lignin-derived aromatic monomers may occur in Nature. Future work will explore the spatial distribution of aromatic catabolism with quantitative proteomics and spatial fluxomics, among other approaches. This represents a novel model for carbon assimilation and transfer within the soil and has implications in both fundamental and applied research areas related to bioenergy and the environment. This work is under preparation for publication as Salvachúa, Werner *et al.*

Reference: [1] Salvachúa et al. *Green Chem* (2015) 17:4951-4967

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