104. Repurposing the Yeast Peroxisome to Compartmentalize Engineered Metabolic Pathways

William C. DeLoache,[1] Zachary N. Russ,[1] **John E. Dueber**[1,2*] (jdueber@berkeley.edu) [1]University of California, Berkeley, [2]Lawrence Berkeley National Labs, Berkeley, California

Project Goals: Engineered metabolic pathways often suffer from undesired interactions with the production host's native cellular processes. Evolution has solved the problem of metabolic crosstalk by segregating distinct cellular functions into membrane-bound organelles. The goal of this project is to repurpose one of these organelles – specifically the peroxisome of *Saccharomyces cerevisiae* – for compartmentalizing heterologous metabolic pathways. Towards this goal, we are working to 1) improve the targeting efficiency of non- native enzyme cargo to the peroxisome, 2) determine the natural chemical composition of the peroxisomal lumen, 3) establish methods for altering this chemical composition to suit the needs of new enzymatic pathways, and 4) demonstrate successful compartmentalization of a model pathway. Ultimately, this work will contribute to the development of a synthetic organelle that can limit metabolic crosstalk and improve production efficiency for a variety of engineered pathways.

We selected the peroxisome as the basis for a synthetic organelle for a few key reasons. First, and most importantly, loss of the peroxisome does not adversely affect *S. cerevisiae* growth provided that long chain fatty acids are not used as the sole carbon source. Second, while peroxisomes occupy only a small fraction of the total cell volume under normal growth conditions, they are capable of expanding dramatically when induced. In some methanol utilizing yeasts such as *Pichia pastoris* and *Hansenula polymorpha*, methanol induction generates massive peroxisomes that often take up more than 70 percent of the total cell volume—an appealing prospect for high-flux engineered pathways. Third, unlike other organelles, the peroxisome's cargo protein is imported directly from the cytosol in the folded state, reducing the probability of protein misfolding for non-native cargo. Finally, many organisms have already repurposed the peroxisome to perform specialized functions—evidence of the organelle's inherent plasticity.

There are two natural targeting tags used for importing protein into the peroxisome: the C- terminal PTS1 tag used by the vast majority of native cargo and the N-terminal PTS2 tag used by just four peroxisomal enzymes. We compared the efficiency of the PTS1 and PTS2 tags by analyzing the peroxisomal import rate of a heterologously expressed enzyme. For our assay, we utilized the bacterial enzyme vioE, which produces the easily detectable green pigment prodeoxyviolacein (PDV). When vioE is expressed in the cytosol of *S. cerevisiae*, it generates large amounts of PDV, resulting in green colonies. However, when sequestered in the peroxisome, vioE is unable to access its substrate, and no PDV is produced. Using this assay, we found that PTS1 import of vioE was many times more efficient than PTS2. In fact, the peroxisomal PTS1 tag proved to be more efficient at sequestering vioE than canonical targeting tags for the vacuole, mitochondrion, or extracellular secretion. Even with a PTS1 tag, however, we still observed detectable levels of cytosolic vioE when the enzyme was expressed at the very high levels utilized by most metabolic engineers. To further improve the import efficiency of PTS1-tagged cargo, we constructed a randomized library of six amino acids preceding the native PTS1 tag and screened for colonies that showed reduced levels of vioE in the cytosol. The output of this screen showed a pronounced trend for improved import when basic residues were preceding the PTS1 tag. Based on these results, we now have a sequence-optimized targeting tag that is capable of importing protein to the peroxisome extremely efficiently. This tag is also modular—we have shown that it maintains its efficiency when fused to a variety of cargo proteins.

In addition to efficient targeting of protein cargo, our strategy demands that we have control over the metabolite pool within the peroxisome. We are addressing this problem in two ways:

1. We are attempting to determine what metabolites are naturally present in the peroxisome and to understand how they get there. Previous studies on the chemical composition of the peroxisome have employed either fluorescent biosensors or purified peroxisomes. We have opted to instead utilize an enzyme-based, *in vivo* approach that is more sensitive than fluorescent biosensors and more physiologically relevant than purified systems. Our assay uses our optimized PTS1 tag to target enzymes of interest to the peroxisome. By comparing enzymatic activity with and without peroxisomal localization, we can determine whether the substrate for each enzyme is present in the peroxisome. Thus far, we have found that a surprisingly high number of small metabolites are present in the lumen of the peroxisome, especially given the existence of only two known metabolite transporters on the membrane of the peroxisome. We believe these metabolites get through the peroxisomal membrane by freely diffusing through a non-specific pore protein—something that has been hypothesized in the literature but has yet to be identified or confirmed *in vivo*. Our current efforts are focused on identifying this pore protein so that we can knock it out in our engineered system.

2. In addition to clearing out metabolites that are natively found in the peroxisomal lumen, we are also developing methods for introducing new metabolites into the lumen via membrane transporters. By fusing the transmembrane segment of a native peroxisomal protein to a variety of plasma membrane transporters, we have demonstrated that we can redirect these transporters to the peroxisomal membrane. The apparent permeability of the peroxisome membrane to small metabolites makes it challenging to assay for the activity of these transporters. Thus, we are currently in search of transporters for larger metabolites that do not get into the peroxisomal lumen natively.

Finally, we are applying our strategy of peroxisomal compartmentalization to a model pathway. We are utilizing a branched pathway in which two enzymes act on the same substrate to generate different products. By producing the shared substrate in the peroxisome, we hope to show that we can control which enzyme acts on it simply be controlling the localization of the downstream enzymes (cytosol vs. peroxisomal). This system mimics a problem that is often encountered in metabolic engineering where a pathway intermediate is lost to a side pathway that happens to exist natively within the cell. We hope that our strategy of peroxisomal compartmentalization will help metabolic engineers overcome this problem as well as many others that arise when engineered pathways are mixed with a production host's native cellular machinery.