

180. Repurposing the Yeast Peroxisome to Compartmentalize Engineered Metabolic Pathways

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Project Goals: Engineered metabolic pathways often suffer from undesirable 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.

We have focused on working with and improving peroxisomal targeting tags by importing 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 C-terminal PTS1 peroxisomal import tag is many times more efficient than N-terminal PTS2 peroxisomal import tag. In fact, the C-terminal PTS1 tag proved to be more efficient at sequestering *vioE* than canonical targeting tags for the vacuole, mitochondrion, or even 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.

To further characterize this improved tag, we used it to evaluate the rate and capacity of protein import of fluorescent proteins and *vioE* using the improved tag. We found that peroxisomal import can be used to

clear a cell of cytosolic fluorescent proteins in an hour, and that 3---10% of a cell's total protein can be encapsulated in unmodified peroxisomes. The improved tag also made possible the encapsulation of a two---enzyme *vioB/vioE* pathway. Finally, we demonstrated use of this modular tag on a number of essential proteins and found the tag enabled inducible growth arrest even when the naïve tag failed. With this enhanced tag, we have achieved efficient, timely targeting of protein cargo. However, our strategy also demands that we have control over the metabolite pool within the peroxisome.

To address this problem, we are attempting to determine which 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, and have conducted experiments to give an approximate measure of peroxisomal permeability. 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.