Deletion of the *ntrYX* two Component System in *Rhodobacter sphaeroides* Causes the Generation of Diverse Extracellular Membrane Structures

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Project Goals: The goals of this project are to understand the process by which bacterial cells produce extracellular membranes in order to engineer strains that overproduce these membranes. Bacterial membranes are a good source of lipids which serve as an important source of primary material for a number of pharmaceutical, industrial, and biofuel applications. In this work, we use the photoheterotrophic bacterium *Rhodobacter sphaeroides* to study membrane synthesis and use cryo-electron microscopy (cryo-EM) to examine how extracellular membranes and other cellular structures are produced. Addressing these goals will provide a significant benefit to the study of renewable biofuels and bioproducts.

Rhodobacter sphaeroides is a facultative photoheterotrophic bacterium that serves as an important host for research into the production of primary materials for industrial purposes and biofuels. Previously, a Tn5 transposon insertion screen, paired with a Nile red assay for lipid production, was used to isolate *R. sphaeroides* strains that overproduce extracellular lipids. By identifying and characterizing the processes that lead to increased lipid secretion in these isolates, this work aimed to broadly achieve production of renewable chemicals and fuels from a biological source. One of the strains, which produces the most extracellular lipids, was disrupted at the *ntrYX* gene locus. NtrY and NtrX comprise elements of a two-component regulatory system known to control exopolysaccharide production, as well as processes induced by respiration and anaerobic growth conditions of other organisms. Deletion of *ntrYX* in *R. sphaeroides* compromises envelope stability and cell division. Cryo-electron tomography (cryo-ET) data supporting those observations will be presented. In addition to the phenotypes caused by *ntrYX* deletion, a diverse array of extracellular membrane structures and chains of vesicles were observed by cryo-EM and cryo-ET, which indicated that there was an increase in the production of extracellular lipids. Our observations demonstrate that these extracellular membraneous structures are closely associated with cells and that their production occurs at the cell surface, consistent with observations that the *ntrYX* disruption causes instability to the bacterial envelope. Tomogram segmentation using EMAN2 neural network training on these membraneous structures will be presented to dissect the physical properties of the extracellular vesicles. Data will also be presented on the observation that cold shock appears to have a synergistic effect on the production of these extracellular vesicles and more complex membrane-derived structures. Future work will determine the molecular mechanisms resulting in cell division defects and instability of the envelope in this and other lipid secreting mutants. By

further understanding the mechanisms by which these *R. shaeroides* strains overproduce lipids, great strides can be made toward creating better chemicals for industrial purposes and biofuels.

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