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

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Rhodobacter sphaeroides is a facultative photoheterotrophic bacterium that is an important model for research into biofuels and the production of primary materials for industrial purposes. In previous work, a genetic screen using a Tn5 transposon insertion library and a Nile red assay for lipid production, *R. sphaeroides* isolates which over produce extracellular lipids were isolated under aerobic growth conditions (1). Determining the mechanisms that lead to the overproduction of lipid secretions in these *R. sphaeroides* isolates may have a broad impact on the production of renewable fuels and chemicals from a biological source. Ten of the highest extracellular lipid producers were analyzed for the loci disrupted through transposon insertion. One of these strains, the highest producer of extracellular lipids, carried an insertion into the ntrYX gene locus. NtrY and NtrX comprise elements of a two-component regulatory system that is known to control exopolysaccharide production and processes controlled by respiration or anaerobic growth conditions (2-4).

R. sphaeroides cultures were grown at 30°C and 200 RPM shaking in Sistron minimal medium until the culture reached an OD600 of 0.6. Suspensions of the cells were then deposited onto 200 mesh R2/1 copper Quantifoil grids in 5 µL aliquots, blotted, and plunge frozen in liquid ethane using a Vitrobot Mark IV (Thermo Fisher). Cryo-electron microscopy (cryo-EM) and cryo-electron tomography (cryo-ET) data were collected using either a Titan Krios TEM (ThermoFisher) operated at 300 kV, equipped with a Gatan Bioquantum energy filter and a K3 direct electron detector (Gatan) or a Tecnai TF30 TEM (ThermoFisher) operated at 300 kV equipped with a Gatan energy filter and a K2 direct electron detector (Gatan). Single axis tilt series were acquired using SerialEM (5), with an increment of 2° covering −60° to +60° and a cumulative dose under 150 e-/Å2 at a defocus range between −4 and −10 µm. Tomograms were reconstructed using IMOD/eTomo (6), and segmentation was performed using the Amira software package (Thermo fisher).

Cryo-EM and cryo-ET were used to determine whether the deletion of ntrYX in *R. sphaeroides* compromises cell division and envelope stability. In addition to these phenotypes caused by ntrYX deletion, an increased production of extracellular lipids was also observed as the production of extracellular membrane structures and vesicles (Fig. 1). These observations correlate with structures previously recovered from concentrated culture supernatants after separating cells from the culture medium by centrifugation (1). Our observations show that these structures are closely associated with cells and that their production occurs at the cell surface, consistent with observations that ntrYX disruption causes instability to the bacterial envelope. Future work will be to determine the molecular mechanisms that result in cell division defects and instability of the envelope. By developing an improved understanding of the mechanisms by which these *R. sphaeroides* strains overproduce cellular lipids, great strides may be made toward creating better biofuels and chemicals for industrial purposes [7].

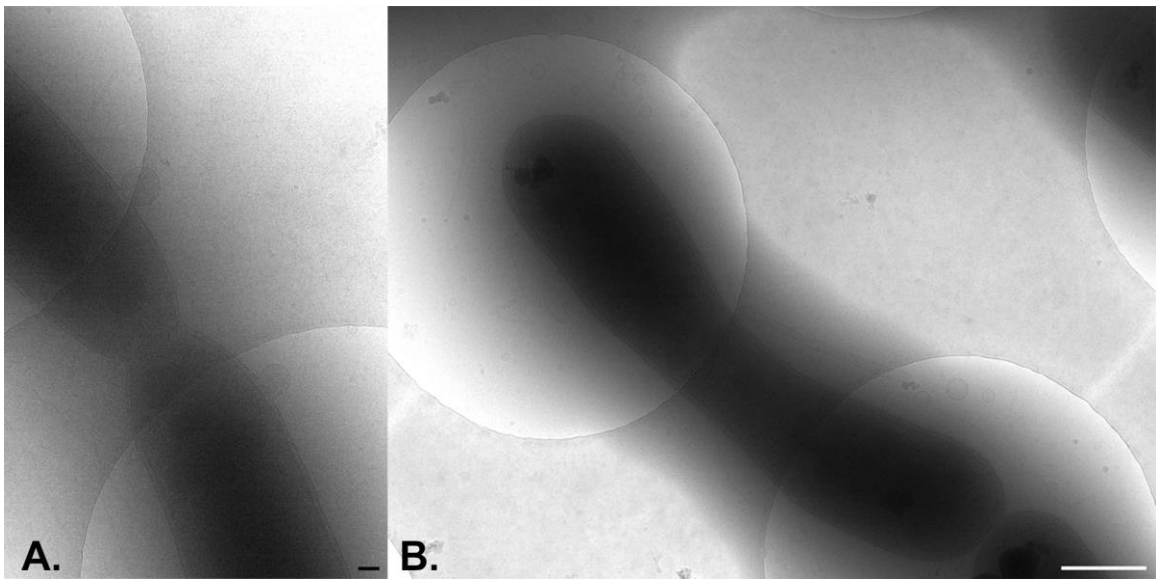

Figure 1. Cryo-EM images of *Rhodobacter sphaeroides* cells. A) An *R. sphaeroides* cell producing membrane vesicles by budding off material from the outer cell membrane. The scale bar represents 100 nm. B) An *R. sphaeroides* cell that has produced membrane vesicles of several sizes, chains of vesicle and a long membrane tubule. The scale bar represents 500 nm.

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