In this collaborative study, we have teamed up with J. C. Liao from UCLA to study and manipulate regulatory networks in phototrophic bacteria to affect and maximize the production of large amounts of hydrogen gas. Our major goal is to understand the factors and regulatory mechanisms that influence hydrogen production. The organisms to be utilized in this study, phototrophic microorganisms, in particular nonsulfur purple (NSP) bacteria, catalyze many significant processes including the assimilation of carbon dioxide into organic carbon, nitrogen fixation, sulfur oxidation, aromatic acid degradation, and hydrogen oxidation/evolution.

The Calvin-Benson-Bassham (CBB) reductive pentose phosphate pathway is responsible for the incorporation of CO_2 into cellular carbon under autotrophic growth conditions. Under photoheterotrophic conditions, CO_2 is primarily used as an electron acceptor via the CBB cycle in order to maintain redox poise within the cell. The key enzyme of the CBB pathway, RubisCO, catalyzes the actual CO_2 reduction step. Over the years we have shown that nonsulfur purple (NSP) photosynthetic bacteria possess an array of metabolic and regulatory capabilities that allow for the utilization of alternative redox sinks when the primary electron sink, CO_2, is nullified via the inactivation or deletion of the RubisCO genes. In order to grow photoheterotrophically, such RubisCO-compromised strains develop interesting strategies and alter their basic metabolic profile. For example, in many instances the derepression of nitrogenase synthesis occurs under normal repressive conditions. Such gain-of-function adaptive mutant strains have been obtained from *Rhodobacter capsulatus, Rhodobacter sphaeroides, Rhodospirillum rubrum*, and *Rhodopseudomonas palustris,* whereby such strains balance their redox potential via nitrogenase-catalyzed reduction of protons to hydrogen gas. Moreover, over the years we have shown that nitrogenase-derepressed mutant strains produce copious quantities of hydrogen gas by virtue of using the nitrogenase enzyme complex exclusively as a hydrogenase. We now show that knocking out competing redox balancing processes such as the CBB pathway plays a crucial role in maximizing hydrogen production in nitrogenase-derepressed strains.

We are in the process of gaining a greater understanding of the molecular basis that allows for the utilization of the nitrogenase complex under normal repressive conditions, particularly in RubisCO-compromised strains. A single point mutation in the *nifA* gene was shown to be important for nitrogenase derepression in RubisCO-compromised mutant strains of *R. capsulatus, R. sphaeroides,* and *R. palustris*. NifA is a key transcriptional activator of the structural genes encoding the nitrogenase complex (*nifHDK*). While current experiments suggest that a mutant NifA protein appeared to be responsible for derepression of the nitrogenase complex in *R. palustris*, in *R. sphaeroides* an additional thus far unidentified mutation appears to be involved in the derepression of the nitrogenase complex. Interestingly, no such *nifA* mutation was found in a nitrogenase-derepressed strain of a RubisCO knockout strain of *R. rubrum*. These results suggest that these organisms utilize different mechanisms to derepress nitrogenase synthesis in CBB-compromised mutant strains. Moreover, derepression of the nitrogenase complex is not the only way in which these organisms may balance their redox potential in the absence of a functional CBB pathway; e.g., some strains may derepress the synthesis of a novel sulfate reduction pathway or use other means to dissipate excess reducing equivalents.

A combined computatational/experimental approach was undertaken with the Liao laboratory [e.g., via the Ensemble Modeling (EM) approach] to examine the behavior of mutant strains. EM was used here to investigate the photoheterotrophic metabolism of *R. sphaeroides* and further verified that inactivation of the CBB pathway rendered the system unable to achieve a redox balance, but was restored through activation of the pathways for either of the above-mentioned routes, while also correctly predicting a significant drop in the uptake rate of the carbon source malate. This work further demonstrated that *R. sphaeroides* is capable of evolving alternative ways to dissipate excess reducing power when the CBB pathway is inactive. Modeling of the system by EM successfully described this behavior and will be very

useful in suggesting further experimental approaches to elucidate molecular mechanisms of control.