

Laboratory reconstruction of ancestral nitrogenase enzymes across an evolutionary transect

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Nitrogen, an essential element for life, has for at least ~2 billion years been primarily supplied to the biosphere by nitrogenases, a unique family of multimeric metalloenzymes that fix nitrogen from atmospheric N₂. Because nitrogen has been a major limiting nutrient for primary productivity over much of Earth history, nitrogenases thus occupy a central role in the evolution of the biosphere. Nevertheless, relatively little is known regarding the origins of nitrogenases, the phenotypic properties of earliest nitrogenase ancestors, and the degree to which environmental geochemistry — namely, the varying concentrations of metals required for nitrogenase activity — have shaped the evolution of this important enzyme family. It is not clear to what extent the features of modern nitrogenases can reliably serve as a proxy for those of their predecessors over billion-year evolutionary timescales. To address this challenge, we have designed an experimental strategy that integrates phylogenetics, synthetic biology, microbiology, and biogeochemistry for the reconstruction of computationally inferred nitrogenase enzymes in the laboratory. Extant genomic data is used to generate an evolutionary model from which the molecular sequences of ancestral nitrogenases can be inferred. We then engineer *Azotobacter vinelandii*, an aerobic, nitrogen-fixing bacterial model, to express ancestral nitrogenase proteins. We have leveraged this experimental system to investigate the impact of these ancestral enzymes, reconstructed across an age transect of nitrogenase evolutionary history, on *Azotobacter* physiology and nitrogen fixation, with the potential to link these features with geochemical nitrogen biosignatures preserved in the geologic record. This approach permits the exploration of evolutionarily informed molecular sequence space to investigate the adaptive constraints on nitrogenase function through time. Moreover, the laboratory reconstruction of ancestral nitrogenases provides a novel strategy to integrate both genomic and geologic records of life in understanding the evolution of the nitrogen biogeochemical cycle across Earth history.