

Contributions to Science:

Contribution 1: Discovery of a new type of carbon pump that drives CO₂ concentration in bacteria:

1.1: Historical background Rubisco is the enzyme responsible for fixing the vast majority of all the CO₂ that enters the biosphere each year and is essential for all plants, algae, and most autotrophic bacteria. However, Rubisco evolved before the great oxygenation event and is competitively inhibited by the presence of O₂. This is a problem that all autotrophs that use rubisco must overcome to survive in the the modern atmosphere, with its 20% O₂ and only 0.04% CO₂. Many autotrophic bacteria including a wide range of chemolithoautotrophs and most cyanobacteria overcome this issue using an α -carboxysome based CO₂ concentrating mechanism (CCM). These CCMs work by creating a compartment inside the bacteria where the concentration of CO₂ is raised high enough to saturate rubisco with CO₂ and out-compete O₂. While our theoretical understanding and previous experimental work suggested that an inorganic carbon (C_i) transporter was absolutely required for the functioning of the CCM, no such transporter was known in the model chemolithoautotroph *H. neapolitanus*.

1.2: Central finding I performed a genome-wide genetic screen for CCM components in *H. neapolitanus*. I identified the essential components of the CCM in *H. neapolitanus*. I cloned putative C_i transporters, called DABs, then confirmed their activity with reporter strain and C_i uptake assays in *E. coli*. I showed that data was consistent with these transporters acting not as direct transporters but as a new family of energy coupled carbonic anhydrase (CA) enzymes. These CAs concentrate C_i by converting membrane permeable CO₂ into membrane impermeable HCO₃⁻ causing a net flow of C_i into the cell. I further showed that similar operons in the human pathogens *V. Cholera* and *B. anthracis* had the same function.

1.3: Influence/Application This work was instrumental in ongoing study of the CCM including a successful effort to reconstitute a functional α -carboxysome CCM in *E. coli*, and work on CCM evolution that I will discuss in contribution 2. Since this work was published, it was shown that a similar operon in *S. aureus* has the same function and is essential for growth in atmospheric CO₂ concentrations. Considering the activity of energy coupled CA transporters has been shown to be necessary to understand the carbon isotope fractionation data in very old rock strata. There has been interest in investigating the potential applications of DABs in engineering crop plants and autotrophic bio-fuel production hosts (like *C. necator*) for increased yield.

1.4: My role I was the primary author on this work. I conceived and designed the experiments, performed the genetic screen, analyzed the sequencing data, did cloning, performed the biochemistry experiments, and performed the reporter strain experiments.

John J Desmarais, ... et al. (Dec. 2019). "DABs are inorganic carbon pumps found throughout prokaryotic phyla". en. In: *Nat Microbiol* 4.12, pp. 2204–2215. ISSN: 2058-5276. DOI: 10.1038/s41564-019-0520-8.

Contribution 2: Characterization of potential evolutionary paths for developing carbon dioxide concentrating mechanisms:

2.1: Historical background The α -carboxysome based CO₂ concentrating mechanism (CCM) required several major evolutionary steps to evolve. These were acquiring uncoupled CA activity, gaining C_i transport, and encapsulating CA and rubisco in an α -carboxysome. However, all of these components are need for the effect of the CCM and removing even one of these components is lethal in modern autotrophs. Since there is no apparent fitness benefit for a partial system, it is not clear how the system could have evolved. Geochemical evidence suggests that the atmosphere was very different when the CCM first evolved, with much higher levels of CO₂ and much lower levels of O₂ and a mixture of biological and geochemical processes has slowly changed the atmospheric composition to the current mixture. This information lead us to the hypothesis that evolutionary intermediates of the CCM may have provided fitness benefits at intermediate atmospheric compositions.

2.2: Central finding We compared the effect of CCM gene knockouts in *H. neapolitanus* across different intermediate CO₂ concentrations. We also compared the growth phenotypes of partial CCM constructs in CO₂ concentration dependent strains of *E. coli* and *C. necator* that we constructed. We found that introduction of an uncoupled CA (non-transporter) or a coupled CA (C_i transporter) provided a fitness benefit at intermediate CO₂ concentrations. We also found that while combining either of these

activities with the α -carboxysome on their own provided no benefit, combining them with each other provided benefits in some situations. This was unexpected, because including a coupled and an uncoupled CA in the same cell without encapsulation is expected to produce a futile cycle. Our mathematical modeling suggests that at intermediate CO₂ concentrations and high growth rates the cell can become limited by both CO₂ and HCO₃ and C_i consumption is high enough that cycling is actually beneficial for providing both C_i species for growth. This revealed a possible path to evolving a CCM through only fitness positive steps by acquiring first either a coupled or uncoupled CA, then acquiring the other type of CA as CO₂ fall further, and finally evolving an α -carboxysome as CO₂ concentrations approach modern levels.

2.3: Influence/Application This work provides insight into the evolution of the CCM and into possible life strategies of modern organisms living in high CO₂ environments. These strategies might also be useful for improving the growth of industrial autotrophs at intermediate CO₂ concentrations. Further, showing the functional expression of the DAB in *C. necator* offers the potential for using the DAB to improve bioplastics production.

2.4: My role My role in this project was to measure the effect of all CCM gene knockouts on the growth of *H. neapolitanus* across a panel of intermediate CO₂ concentrations to establish which components of it's CCM are needed for growth at intermediate CO₂ concentrations.

Avi I Flamholz, . . . , John J Desmarais, . . . et al. (2022). "Trajectories for the evolution of bacterial CO₂-concentrating mechanisms". In: *Proceedings of the National Academy of Sciences* 119.49, e2210539119. DOI: 10.1073/pnas.2210539119.

Contribution 3: Development of nuclease amplification for cas13 viral diagnostics:

3.1: Historical background CRISPR based diagnostics offer an attractive option for rapid point of care or at home detection of nucleic acids, such as virus genomes. Their advantages include fast detection times, ease of conversion into either lateral flow or fluorescence assays, and simple operation (they do not require multiple liquid handling or incubation steps). However existing CRISPR diagnostics were not sensitive enough to detect clinically relevant levels of SARS-COV2 in patient samples without pre-amplification of target sequences which negated all of these advantages. Class III CRISPR systems include a cyclic-oligo-A activated nuclease csm6 that is used to amplify the CRISPR immune response. We were interested in determining if we could link cas13 detection of viral RNAs to csm6 activation to improve limit of detection and allow SARS-COV2 detection in clinical samples.

3.2: Central finding We found that cleavage of an A4-U6 substrate by cas13 produced a good activator for csm6. However, the reaction appeared to be self limiting. Kinetic modeling and reagent spike in experiments suggested that csm6's intrinsic activator cleavage activity was causing the self-limitation. By using a single-fluoro modified activator, we were able to remove this self limitation and detect SARS-COV2 genomes in clinical samples. We also developed a microfluidic device for automating sample processing and assay performance.

3.3: Influence/Application This work has demonstrated a new method of improving time to detection and sensitivity in CRISPR diagnostics. This work will contribute to new and improved diagnostics technologies for the detection of a variety of clinically and scientifically relevant nucleic acids.

3.4: My role My role in this work was to perform kinetic modeling of different potential reaction setups to predict potential improvement in time to detection or limit of detection, this included the analysis that suggested that using an uncleavable activator would remove self-limitation. I also wrote analysis pipelines to process data and perform statistical analysis of data, including developing methods for detecting positive samples from microfluidic device data.

Tina Y Liu, . . . , John J Desmarais, . . . et al. (Aug. 2021). "Accelerated RNA detection using tandem CRISPR nucleases". en. In: *Nat. Chem. Biol.*, pp. 1–7. ISSN: 1552-4450. DOI: 10.1101/2021.03.19.21253328.

Contribution 4: Application of general epistasis techniques to protein design:

4.1: Historical background

4.2: Central finding

4.3: Influence/Application

4.4: My role

Contribution 5: Discovery of CasX:

5.1: Historical background

5.2: Central finding

5.3: Influence/Application

5.4: My role

Jun-Jie Liu, ..., John Desmarais, ... et al. (Feb. 2019). “CasX enzymes comprise a distinct family of RNA-guided genome editors”. en. In: *Nature*, p. 1. ISSN: 0028-0836. DOI: 10.1038/s41586-019-0908-x.

Contribution 6: Improved production of chemicals in E. coli through nitrogen limitation:

6.1: Historical background

6.2: Central finding

6.3: Influence/Application

6.4: My role

Victor Chubukov, John James Desmarais, ... et al. (Jan. 2017). “Engineering glucose metabolism of Escherichia coli under nitrogen starvation”. In: *NPJ Syst Biol Appl* 3, p. 16035. ISSN: 2056-7189. DOI: 10.1038/npjbsa.2016.35.