Exploring the Mechanisms of Intracellular Calcium Carbonate Biomineralization in Cyanobacteria: A Microbiology, Genetics, and Microscopy Approach

Cyanobacteria have long been recognized for their role in inducing $CaCO_3$ precipitation and contributing to the formation of limestones, such as stromatolites, over geological time. The prevailing paradigm was that this occurs through extracellular precipitation, driven by the metabolic activity of cyanobacteria, in a genetically unregulated manner (Görgen et al., 2021). However, this view was challenged by the discovery of certain cyanobacteria capable of biomineralizing intracellular amorphous calcium carbonates (iACC), using specific genes (Couradeau et al., 2012; Benzerara et al., 2014 & 2022). Since this discovery, iACC biomineralization has been observed in an increasing diversity of microorganisms across diverse environments (Mangin et al., 2025). Moreover, because iACC inclusions can accumulate large amounts of calcium, strontium, barium and radium, including their radioactive isotopes, these cyanobacteria show potential for innovative water bioremediation strategies (Mehta et al., 2022; Pamart et al., in rev.). Despite its biological and environmental significance, the underlying biological function of iACC biomineralization remains poorly understood particularly with regard to the mechanisms of calcium homeostasis (De Wever et al., 2019), highlighting the need for further dedicated research.

Comparative genomics of iACC-forming cyanobacteria vs. species lacking iACC has led to the identification of a novel gene family, named *ccyA*, encoding a protein called calcyanin. This protein has been established as a diagnostic marker for iACC biomineralization (Benzerara et al., 2022). By tracking the *ccyA* gene in available genome databases, a diversity of cyanobacteria capable of iACC biomineralization has been uncovered, revealing a broader phylogenetic and environmental distribution than previously thought. This includes various unicellular strains (*Gloeomargarita*/*Synechococcus*/*Cyanothece*/*Chroococidiopsis*), as well as clades like *Fischerella*, filamentous strains with differentiated cells and branching, or *Microcystis*, toxic colonial unicellular organisms that proliferate in freshwater ecosystems worldwide (Gaëtan et al., 2022), emphasizing the environmental significance of this process. Overall, iACC inclusions have been detected in > 50 strains of cyanobacteria, including those preserved for over 60 years in the Pasteur cultures of Cyanobacteria (PCC) collection. Moreover, the comparison of species phylogeny with that of the *ccyA* gene supports the hypothesis of an ancestral origin, possibly over a billion years old. The function of the calcyanin protein remains unknown, but recent bioinformatics analyses suggest it may interact with other proteins and exhibit an affinity for lipids (Gaschignard et al., 2024). Preliminary genetic analyses also suggest that calcyanin plays a pivotal role in Ca homeostasis (Benzerara et al., 2022). In addition to be involved in Ca homeostasis, iACC may serve as intracellular reservoirs of inorganic C, which is crucial for the metabolism of cyanobacteria. However, the relationship between iACC formation and C or Ca availability has not been quantitatively tested and the factors that regulate the production of iACC remain unclear. Finally, genomic and transcriptomic analyses of *Microcystis aeruginosa* PCC 7806 revealed that the *ccyA* gene is expressed at the end of the night and co-expressed with adjacent genes, including one that codes for a Ca^{2+} transporter, suggesting a functional unit for these genes (Bruley et al., 2025). This genomic arrangement is also found in other cyanobacteria that form iACC, though the functional implications remain to be experimentally validated. These findings support the idea that the *ccyA* gene alone is insufficient for iACC formation and that this process likely involves additional, yet unidentified genes.

This thesis project aims to combine microbiology, genetics, and advanced microscopy to (i) cultivate a variety of iACC-forming strains, quantify the minerals formed, and characterize them to determine how environmental conditions (e.g., day/night, partial CO_2 pressure, Ca^{2+}

availability) impact the amount of iACC formed; (ii) study, using transcriptomics (RNAseq), the expression of genes involved in biomineralization under different conditions and determine if expression of some genes (*ccyA* and/or adjacent or other genes) correlates with the rate of biomineralization; and (iii) inactivate certain of these genes using a CRISPR-based approach to confirm their functional involvement in the biomineralization process of iACC.

The thesis project will be conducted jointly at (i) IMPMC under the supervision of Karim Benzerara in Sorbonne University and (ii) the collection of cyanobacteria under the co-supervision of Muriel Gugger at Institut Pasteur.

Cultures of a diversity of iACC-forming strains will be performed under controlled and high pCO_2 using the CellDeg photobioreactor, available at Institut Pasteur. The possibility to have these strains grow at high cell densities will be systematically tested as some strains seem to respond positively while others do not. Then, on selected strains, the student will vary cultures conditions such as Ca concentration (low vs high), pCO_2 and day/night cycles. In parallel, the PhD student will conduct exhaustive chemical analyses and modelling of the culture solutions at the GEMME platform at IMPMC to quantify chemical element uptake, in particular Ca consumed by iACC formation. She/he will also conduct infrared spectroscopy and scanning electron microscopy to further quantify the amount of iACC formed. Overall, biomineralization rates will be quantified under these different conditions. This experiment will be conducted over six months and will produce the biomass for the second step.

In a second step, on a selected set of strains and conditions, the student will conduct RNAseq experiments to measure gene expression under different conditions (collab. with BIOMICS). The student will follow the approach already set in an IMPMC-Pasteur collaboration on *Microcystis* PCC 7806 (Bruley et al., 2025). Nucleic acid extraction and RNA sequencing will be performed at Institut Pasteur. Read processing, gene functional annotation and sequence and structure analysis of potential unknown proteins will be performed in collaboration with Isabelle Callebaut at IMPMC. Overall, this will allow to test the existence of correlations between the overexpression of some genes (e.g., *ccyA*; neighboring genes; others….) and the high production of iACC. This *in silico* phase will be performed during ~1 year.

The last year, a CRISPR-based approach will be conducted by the PhD student to test the functional involvement of these genes in the biomineralization of iACC. CRISPR/Cas12a has already been successfully used to delete *cax* genes (Ca^{2+}/H^+ transporter) in the iACC-forming cyanobacterium *Cyanothece* sp. PCC 7425 (unpubl.). Genetics experiments will be performed at Institut Pasteur. Analysis of the phenotype of the mutant strains will be conducted using aqueous chemistry measurements as well as confocal laser scanning microscopy, scanning and transmission electron microscopies and infrared spectroscopy at IMPMC.

References (*first author is a student co-advised by one or both of us)

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