Doctoral Thesis Project: Study of interactions between organic molecules and amorphous calcium carbonate in cyanobacteria

Cyanobacteria are widespread, environmentally important photosynthetic organisms. They capture atmospheric CO_2 using solar energy, and they participate to the production of calcium carbonates ($CaCO_3$) This mineral represents more than 4% of Earth's crust and its mostly produced by biomineralization[1]. The process of biomineralization is defined as the formation of hybrid organic-inorganic minerals in a controlled manner by living organisms. Biominerals are produced in every phylum of every kingdom of life but, while many cases of biomineralization in eukaryotes involve specific genes, for many years only intracellular magnetite formation by magnetotactic bacteria was known as an example of controlled intracellular biomineralization in prokaryotes.

Since 2012, intracellular amorphous calcium carbonate (iACC) inclusions have been observed by the team in tens of cyanobacterial strains[2]. Formation of iACC was observed in many different environments, including some where the formation of these mineral phases is thermodynamically unfavorable, suggesting that this process comes with some energy cost. The role of calcium in intracellular signaling, gene expression and cell differentiation processes is well documented in eukaryotes, whereas it remains poorly understood in prokaryotes. Nevertheless, we could show that a single cell is able to concentrate a high amount of calcium in iACCs, equivalent to a concentration in the molar range if released to the whole cell. Moreover, some species accumulate very high calcium contents but also other alkaline earth elements (AEEs) such as strontium (Sr), barium (Ba) and radium (Ra), which replace calcium in iACCs. One of the intriguing hypotheses concerning the role of intracellular iACCs is that they act as inorganic pH-buffers or calcium and/or carbon storage. But the benefit for cyanobacteria to store such high amounts of alkaline earth elements, which moreover comes with some energy cost, still needs to be elucidated.

Abiogenic ACC is relatively unstable while the one present in bacteria stays amorphous all along the cell's life and does not turn to a crystalline phase. How cells can keep calcium carbonate in a stable amorphous phase is still unclear. In iACC forming cyanobacteria, cryo-ultramicrotomy (CEMOVIS) allowed to visualize envelopes around the iACC in wild-type strains, even though the nature of the envelop was not defined[3]. We also described the local structure of iACC directly in cells using X-ray absorption spectroscopy (XAS) at the bulk scale. This allowed to show that at least on average at this scale, the calcium coordination environment in cyanobacterial ACC was different from that of abiotic stable ACC and that the stability might be related to hydration level of ACC and/or the action of biological macromolecules and additives such as magnesium or phosphorous (Mehta et al., in preparation).

A comparative genomics analysis showed that one single gene family, with no homolog with known function, was found in iACC-forming cyanobacteria and absent in the others[4]. We named this gene, ccyA, and it encodes a protein named calcyanin. Phylogenetic analyses suggest that ccyA has a very ancient origin in cyanobacteria, supporting the hypothesis that the associated biomineralization phenotype is an important physiological process. Calcyanin is composed of two domains: a conserved C-terminal domain composed of a large glycine-rich motif repeated 3 times ((GlyZip)3); and variable N-terminal domains, named X-, Y-, W- and Z-type. The W type domain, is the most widespread among iACC-forming cyanobacteria and is the only one sharing sequence similarities with three already known families of the HMA superfamily (Heavy-Metal-associated domains) often found associated with P-type ATPases. However, the domain in calcyanin has a specific sequence signature with several basic aminoacids and an extra N-terminal β-strand at the beginning of the thioredoxin fold, suggesting that it may have a specific function. Therefore, we named this novel domain family, of unknown function, CoBaHMA (Conserved Basic residues in the HMA superfamily). Additionally, genetically modified cyanobacteria overexpressing ccya had higher Ca contents, suggesting that calcyanin may somehow impact calcium homeostasis and/or

iACC formation. The identification of a biomineralization gene marker opened the door to both an experimental work on structural characterization of the CoBaHMA domain and a bioinformatics analysis of the CoBaHMA family of domains (Gaschignard et al. in preparation). However, there is now a crucial need to tackle the question of calcyanin localization in the cells, its potential interaction with iACC, and how organic molecules (calcyanin or other ones) may interact with iACC.

Working hypotheses and objectives

The main goal of this PhD project is to combine biochemistry and advanced microscopy and spectroscopy tools in order to locate calcyanin within cyanobacteria and characterize the structure and chemistry of iACC, including its association with organic molecules down to the nanoscale.

We can raise two main hypotheses concerning the role of calcyanin that need to be tested: first, calcyanin may play a direct role in the stabilization of iACC in cells. This may occur by forming a confining envelope around iACC granules and/or being embedded within iACC inclusions and trigger stabilizing interactions. Alternatively, calcyanin may be localized away from the iACC inclusions. In this case, depending on its localization, it may be involved in calcium or carbonate transport or serve as a gene activation factor. In this second hypothesis, other organic molecules, such as proteins, polysaccharides or lipids may play a role in the stabilization of iACC. Alternatively, the inorganic composition or degree of hydration of iACC may explain their stability compared with abiotic ones.

More precisely, the PhD candidate will focus on two related objectives:

1 the characterization of iACC at the atomic and nanoscale and the nature of the envelope

Before describing the molecular architecture of iACC at the atomic level, the PhD student will first work on synthetic model ACC[5] which will serve to setup protocols, calibrate analyses but also evaluate the role of macromolecules such as proteins (including CoBaHMA, GlyZip3 and the full calcyanin protein), lipids, magnesium and phosphate on the morphology, phase stability and size during formation process.

Then, the PhD student will extract iACC from cells of several strains and identify involved proteins. More precisely, strains will be cultured in the BG11 medium with calcium. Cells will be disrupted using a Beads beater lysing system. Lysates will be clarified and fractioned by centrifugation testing various organic solvents. Samples of each fraction will be used to prepare cryo-EM grids to verify the presence of iACC. iACC will be analyzed to search for residual organic molecules, using SDS page and mass spectrometry. The PhD student will also use TEM to characterize the chemical and structural properties at iACC at the sub nano scale and localize organic molecules associated with ACC (either at their surface and/or within the inclusions). In both cases, i.e. for synthetic ACC and iACC, structural analyses will be achieved using Cryo-electron Pair Distribution Function (Cryo-ePDF) to get information on their local structure[6]. This method is particularly suitable for amorphous or poorly crystallized particles such as the intracellular iACC since it preserves fragile structures and improves data quality. For synthetic ACC, available in large quantity, X-ray PDF and Cryo-ePDF results will be compared to assess the validity of the approach. In a second step, the localization of calcyanin protein will be attempted using Electron Energy Loss Spectroscopy at ultra-high energy resolution using the METSA facility and possibly the future PANAM facility of Sorbonne Université. The ability of EELS to detect and identify functional groups in organic biomolecules in the near infra-red energy range with marginal damages has been demonstrated for guanine[7].

2 the structural characterization of calcyanin and its role in iACC stabilization

The PhD student will take advantage of our strong expertise in biochemistry to achieve the structural characterization of the ((GlyZip)3 domain by X ray crystallography and the full length protein by cryo-electron microscopy. Moreover, purified proteins will be used to measure the affinity of calcyanin for AEEs. For this purpose, full-length proteins and domains will be studied using fluorescence spectroscopy, Trp fluorescence and isothermal titration calorimetry to analyze and compare the binding properties of Ca, Sr and Ba to the protein. He will also test the affinity of calcyanins for lipids using lipid strips and the associations with membranes using reconstituted liposomes (with different lipid compositions) in cryo-EM.

In parallel, she/he will assess the spatial distribution of calcyanin within cells of a mutant strain of the cyanobacterium *Cyanothece* sp. PCC 7425, which overexpresses the *ccyA* gene. For this purpose, the student will set up protocols for expansion microscopy[8] in order to increase the size of the cells and allow an easier detection of calcyanin staining by a fluorescent antibody that we recently produced and tested. Expansion microscopy is a promising approach that has already been used on bacteria[9] and will be performed testing various conditions for fixation but also the cryo fixation approach, recently implemented in Curie cryo-electron microscopy platform.

During the first year, the PhD student will conduct biochemistry, structural biology and optical microscopy analyses. She/he will focus on optimizing proteins expression and stability. Once we will have purified proteins, he/she will launch crystallogenesis and sample preparation for cryo-EM and start testing condition for expansion microscopy, thanks to the facilitated access to the crystallography platform and also the 200 kV Glacios Cryo-Electron Microscope at the Curie Institute and the 300 kV Titan Kryos in Pasteur Institute. In parallel, she/he will optimize protocols for extracting intracellular carbonates inclusions and also the synthesis of ACC in presence of organic/inorganic molecules. This will be continued in second year until he has exploitable XRD results and cryo-EM pictures and start functional test for calcium binding. In parallel during the second year, he will investigate the structure and chemistry of iACC down to the nm-scale, starting with synthetic and then purified iACC. Finally, the third year will be dedicated to finalize the structural analysis, and data concerning iACC characterization and to write the papers and the thesis manuscript. The joint supervision by Prof Nicolas Menguy, a specialist in material science and transmission electron microscopy and Manuela Dezi and Fériel Skouri-Panet, specialists in biochemistry and structural biology will provide a unique opportunity for the student to be trained interdisciplinary.

Bibliography

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