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## **Intracellular formation of carbonates by bacteria as a new case of genetically controlled biomineralization**

Karim Benzerara, Khan Monis Athar, Isabelle Callebaut, Elodie Duprat, Geoffroy Gaschignard, Christopher T. Lefèvre, Camille Mangin, Neha Mehta, Nicolas Menguy, Caroline Monteil, et al.

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Karim Benzerara, Khan Monis Athar, Isabelle Callebaut, Elodie Duprat, Geoffroy Gaschignard, et al.. Intracellular formation of carbonates by bacteria as a new case of genetically controlled biomineralization. MTM2024 - 8th International Meeting on Magnetotactic Bacteria, Jun 2024, Bilbao, Spain. hal-04871270

**HAL Id: hal-04871270**

**<https://hal.science/hal-04871270v1>**

Submitted on 7 Jan 2025

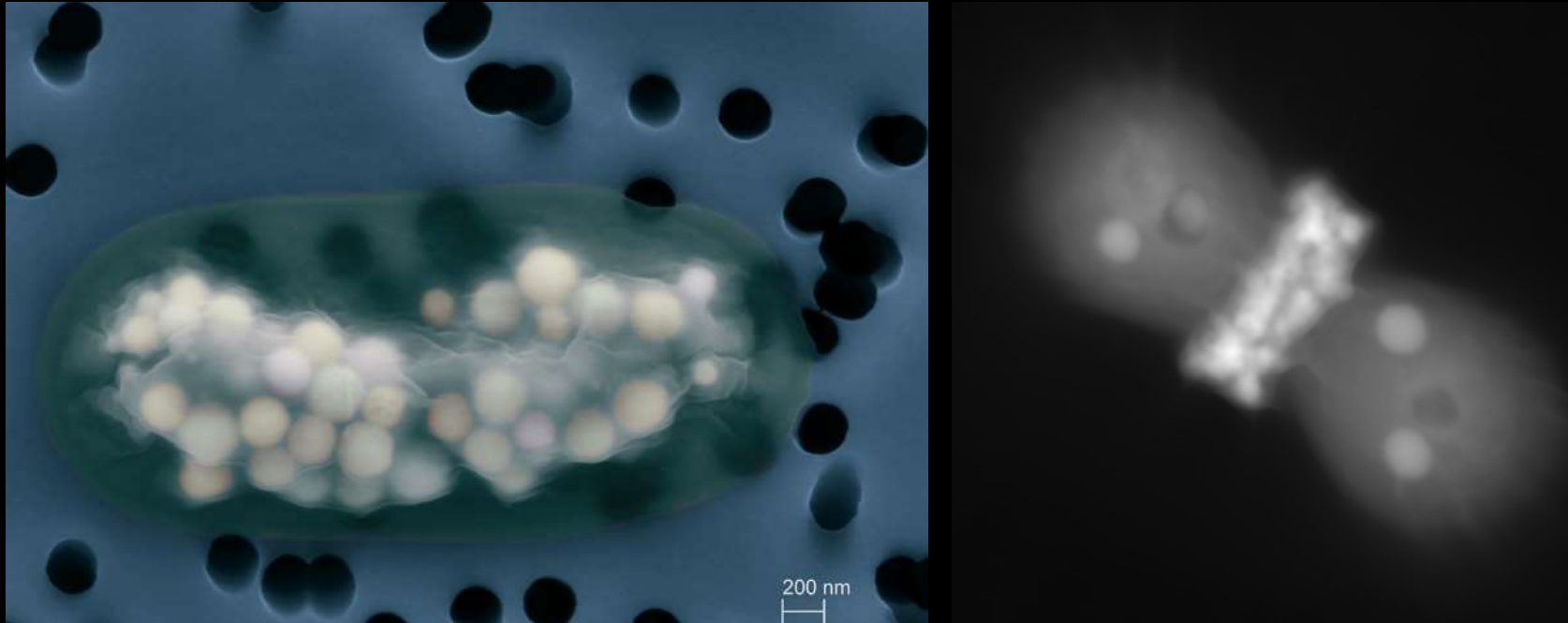
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# Intracellular formation of carbonates by bacteria as a new case of genetically controlled biomineralization



Karim Benzerara, Khan M. Athar, Isabelle Callebaut, Elodie Duprat, Geoffroy Gaschignard, Christopher T. Lefevre, Camille Mangin, Neha Mehta, Nicolas Menguy, Caroline L. Monteil, Fériel Skouri-Panet, ANR Carbomagnet and the ANR Harley consortia

Institut de Minéralogie et de Physique des Milieux Condensés (IMPMC)  
CNRS, Sorbonne Université & Muséum National d'Histoire Naturelle

Bacterial biomineralization is often considered as a non controlled mineralization process (except for MTB)



Modern stromatolite formed in Sardinia

*Debie et al. (2022) Chem Geol*

e.g., extracellular  $\text{CaCO}_3$  precipitation by cyanobacteria



Serendipitous discovery of a cyanobacterium forming intracellular amorphous  $\text{CaCO}_3$  in a controlled manner

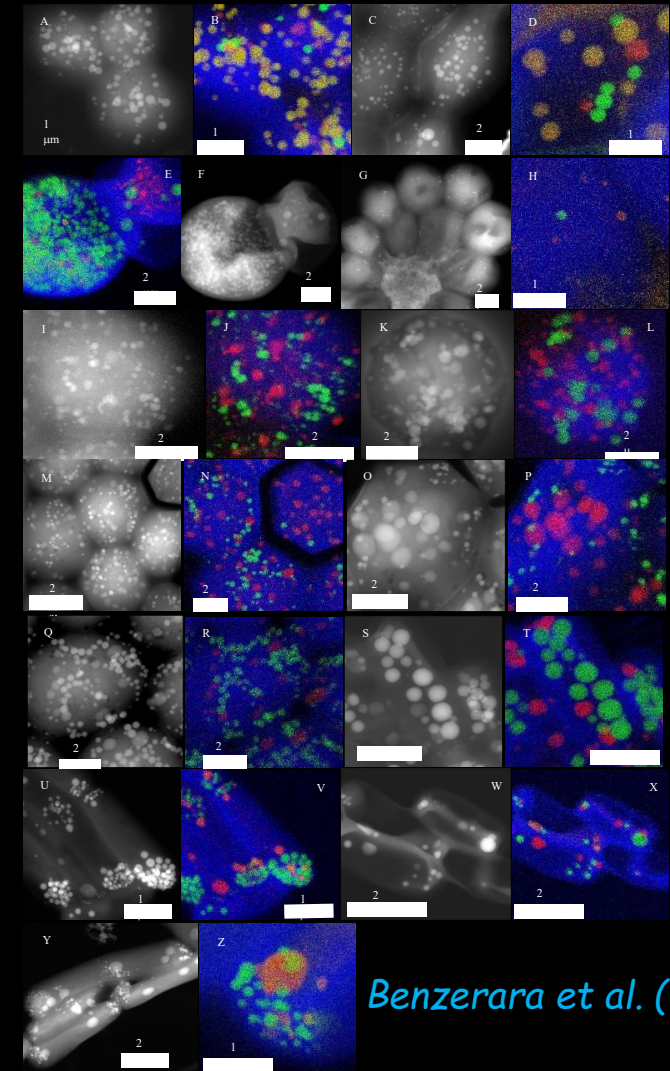
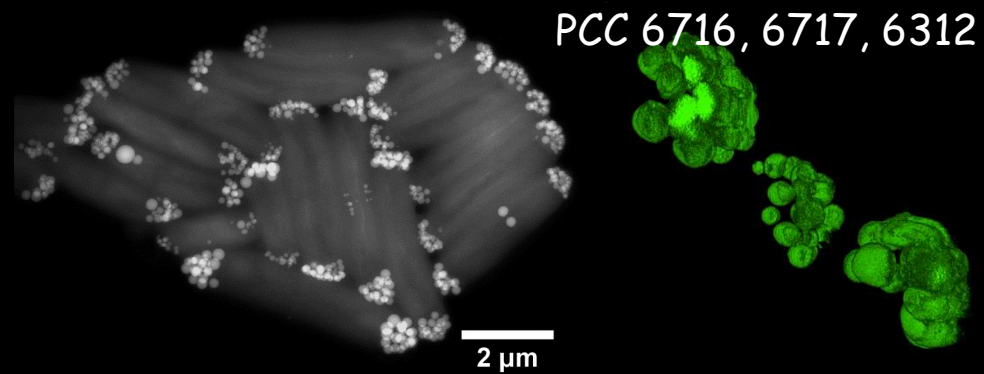
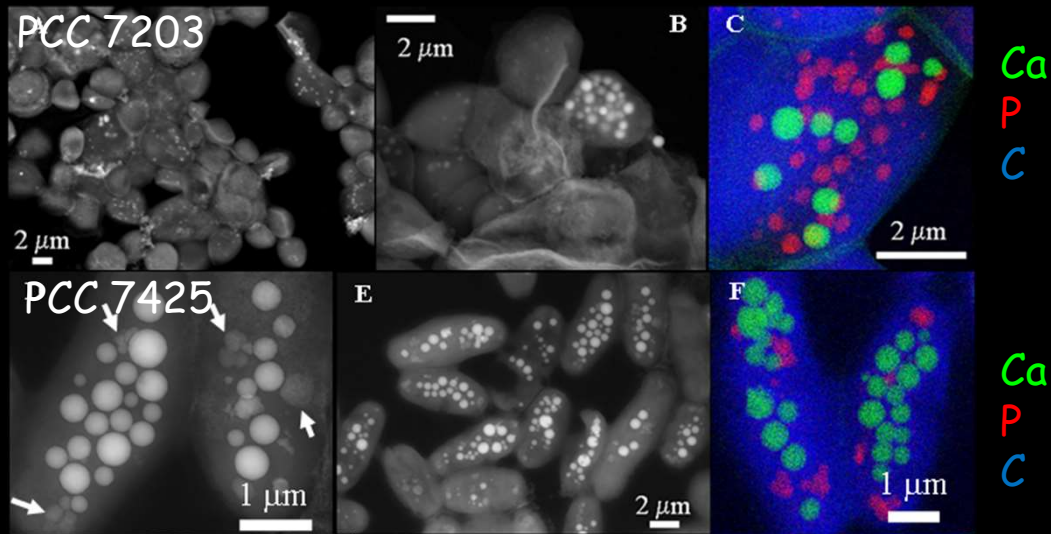
→ *Gloeomargarita lithophora*



*Couradeau et al (2012) Science*

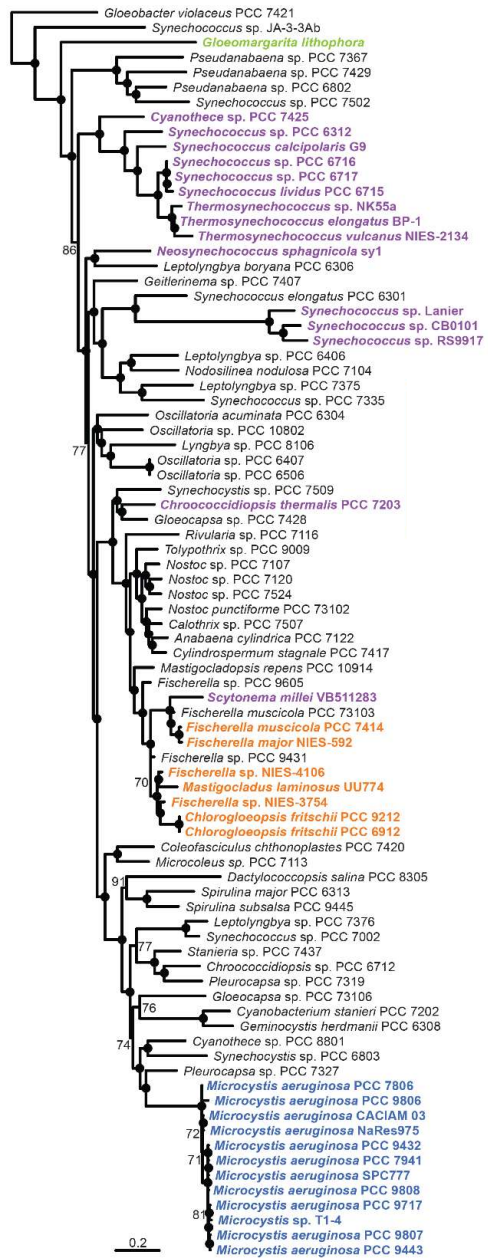


# Now hundreds of cyanobacteria from diverse families known to form intracellular ACC



*Benzerara et al. (2022) GBE*

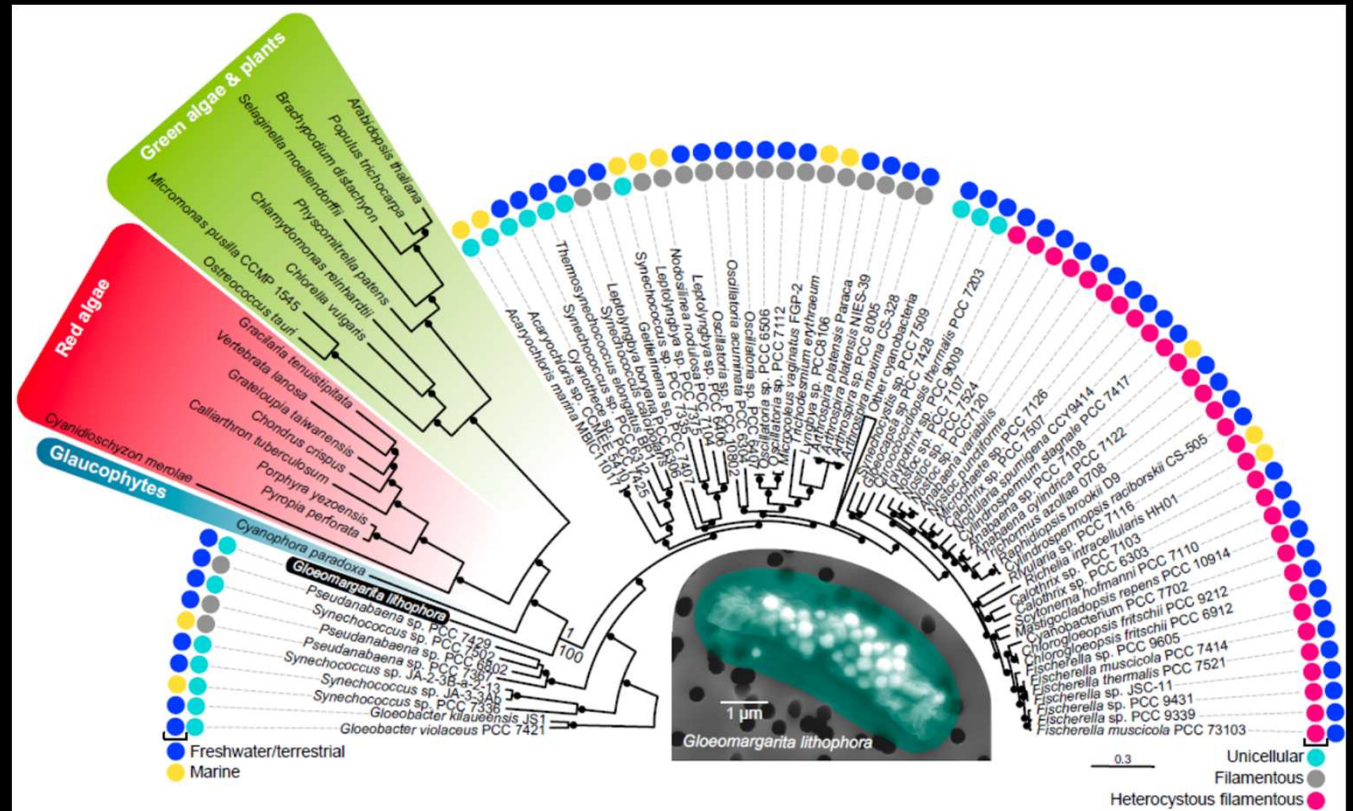
Single cell cyanobacteria; Multicellular cyanobacteria; Marine cyanobacteria ...



## Widespread distribution of iACC-forming cyanobacteria in phylogenetic tree

Indications that intracellular biomineralization is ancestral in Cyanobacteria

*G. lithophora* is the phylogenetically closest extant cyanobacterium to plastids



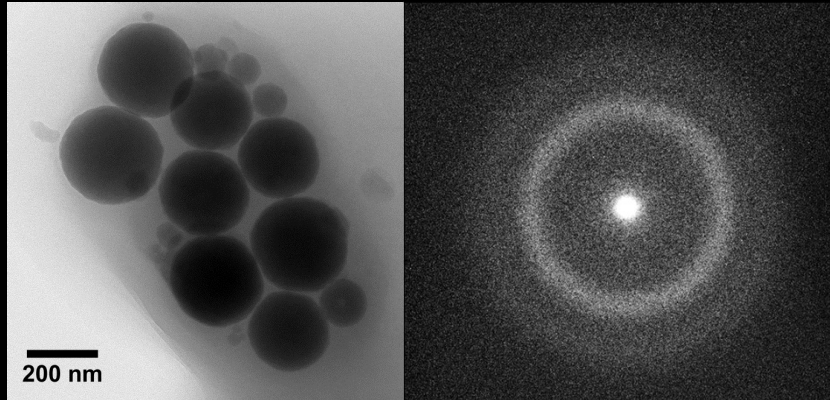
Ponce-Toledo et al (2017) Current Biology

Was the formation of intracellular CaCO<sub>3</sub> transferred to photosynthetic eukaryotes ?



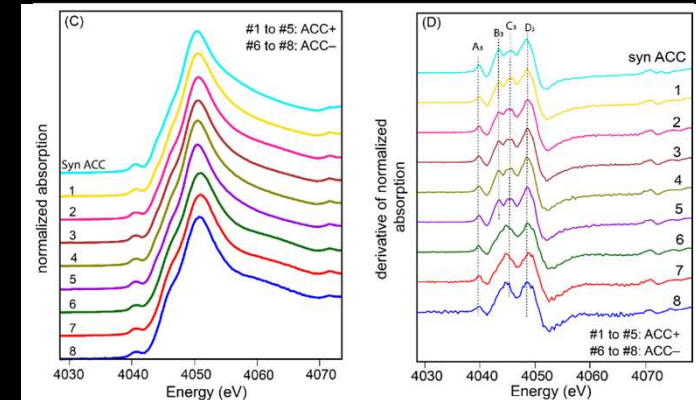
# Amorphous calcium carbonates (ACC)

TEM



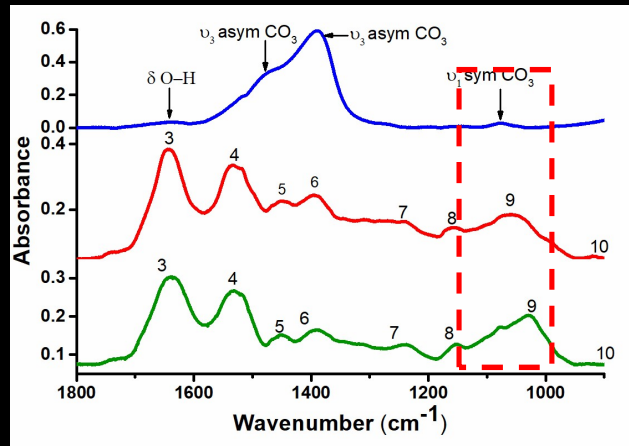
Li et al. (2016) Minerals

XAS at the Ca K-edge



Mehta et al. (2023) Chem Geol

FTIR



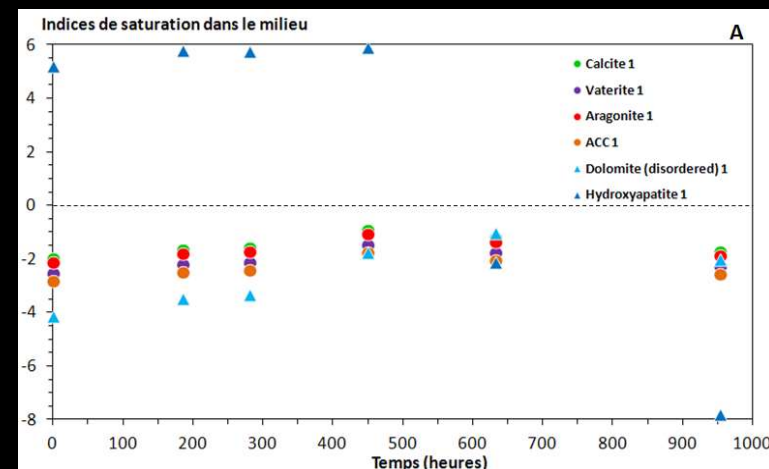
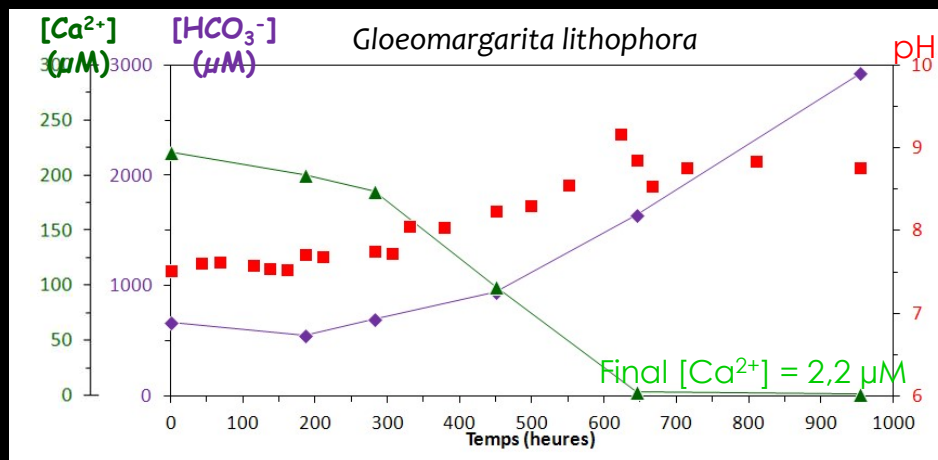
Mehta et al. (2022) Spectrochimica Acta

- █ Synthetic ACC
- █ ACC-forming cyanobacteria
- █ Non-ACC-forming cyanobacteria

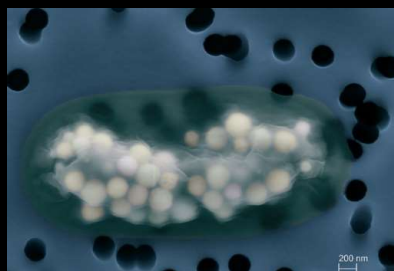
They seem to stay intracellular and amorphous in a culture

## Apparent out-of-equilibrium process

Formation of intracellular  $\text{CaCO}_3$  in environments undersaturated with  $\text{CaCO}_3$  phases



Fate of intracellular ACC after cell death?

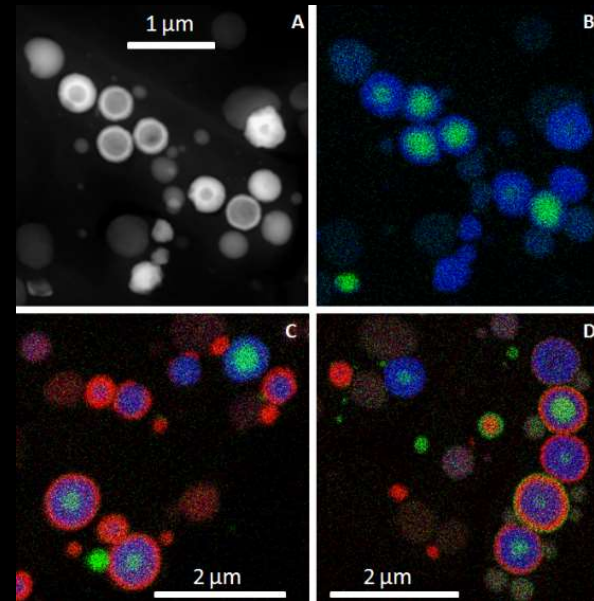
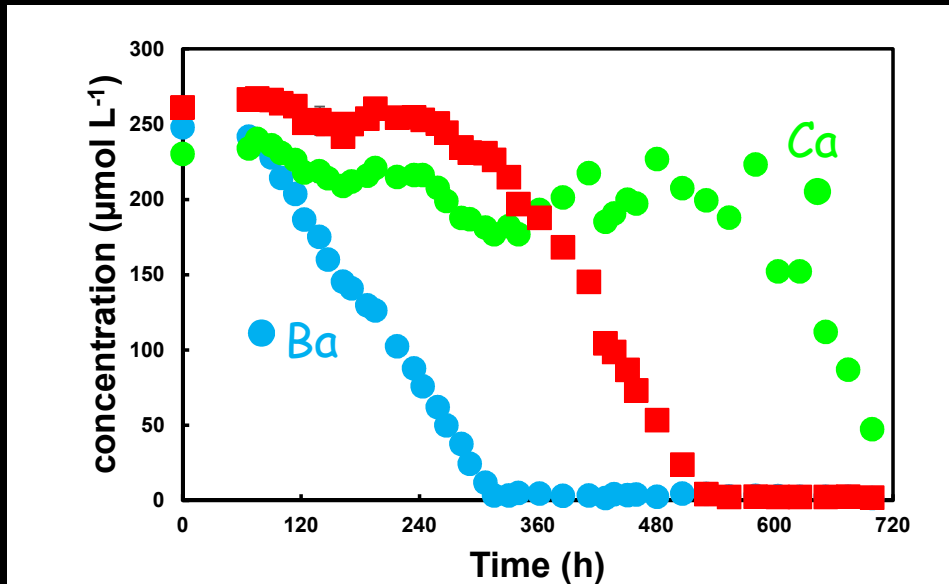


Extracellular  $SI_{\text{calc}} < 0$ : dissolution (?)

Extracellular  $SI_{\text{calc}} > 0$ : crystallization (?)

# Unusual element fractionation by, e.g., *Gloeomargarita lithophora* = preferential uptake of high-Z alkaline earth metals

Cam et al. (2016) ES&T; Blondeau et al. (2018) Chem Geol.



Ba > Sr > Ca

Cam et al. (2016) ES&T

→ Formation of Sr and/or Ba-rich ACC despite inconsistent environmental chemical conditions

= singularity of intracellular carbonates



# Some of these bacteria may help remediate radioactive pollutions ( $^{90}\text{Sr}$ and $^{226}\text{Ra}$ )

→ efficient trapping of heavy alkaline earth metals, incl. radioactive isotopes

nature Subscribe

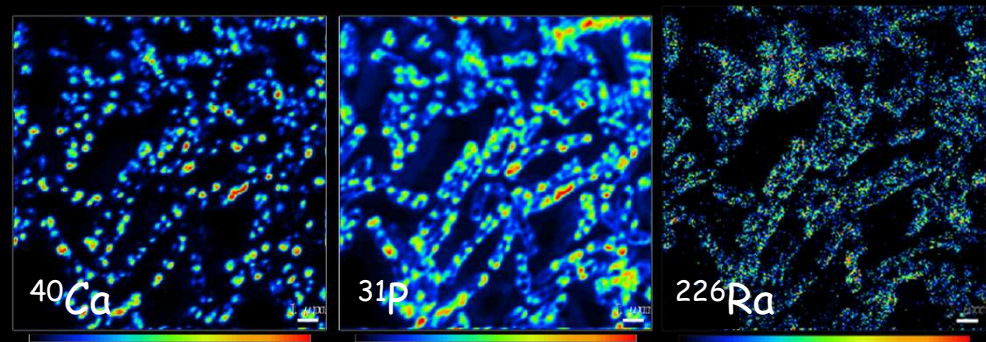


A *Gloeomargarita lithophora* bacterium containing carbonate granules (white spheres). The microbe's ability to form such granules internally might explain how it draws radioactive isotopes from its surroundings. Credit: Karim Benzerara and Stefan Borensztajn/CNRS

ENVIRONMENTAL SCIENCES · 15 OCTOBER 2019

### Nuclear waste? These microbes might help with the cleanup

Bacteria discovered in a Mexican lake accumulate two radioactive isotopes in their cells.



NanoSIMS mapping of Ca, P, Ra

$250 \mu\text{M}$  of Ca +  $9 \cdot 10^{-5} \mu\text{M}$   $^{90}\text{Sr}$  (or Ra)

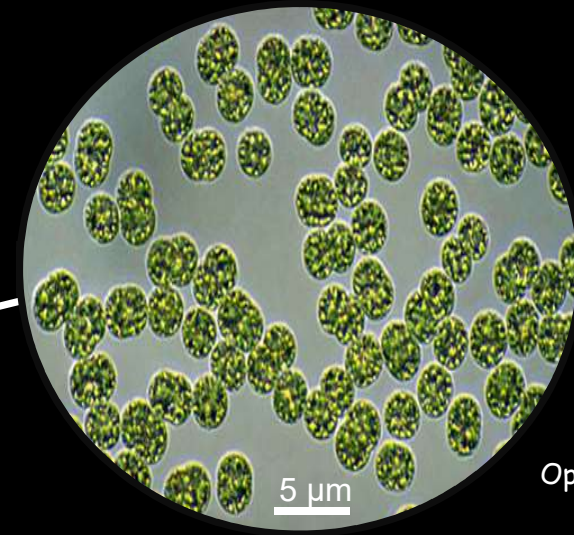
Mehta et al (2019) *ES&T*  
Mehta et al (2022) *ES&T Water*

# Some of these ACC-forming cyanobacteria form blooms (high cell densities)

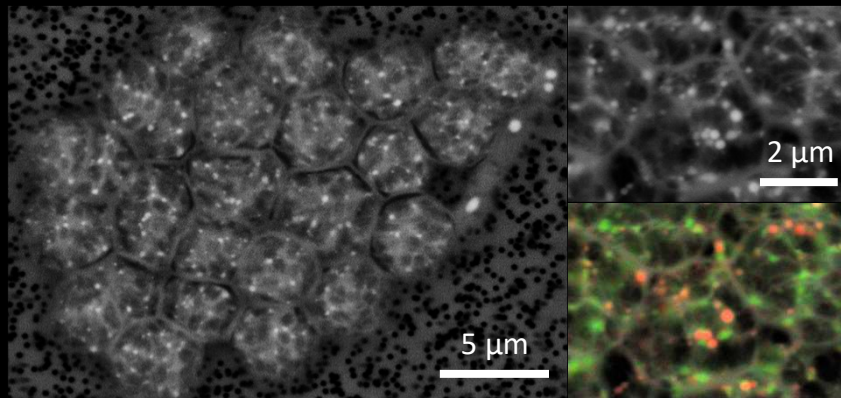
→ cyanobacterium blooming in eutrophic aqueous environments  
- major health/environmental issue



*Microcystis* bloom (Maimee River, Ohio, USA) @David J.Ruck



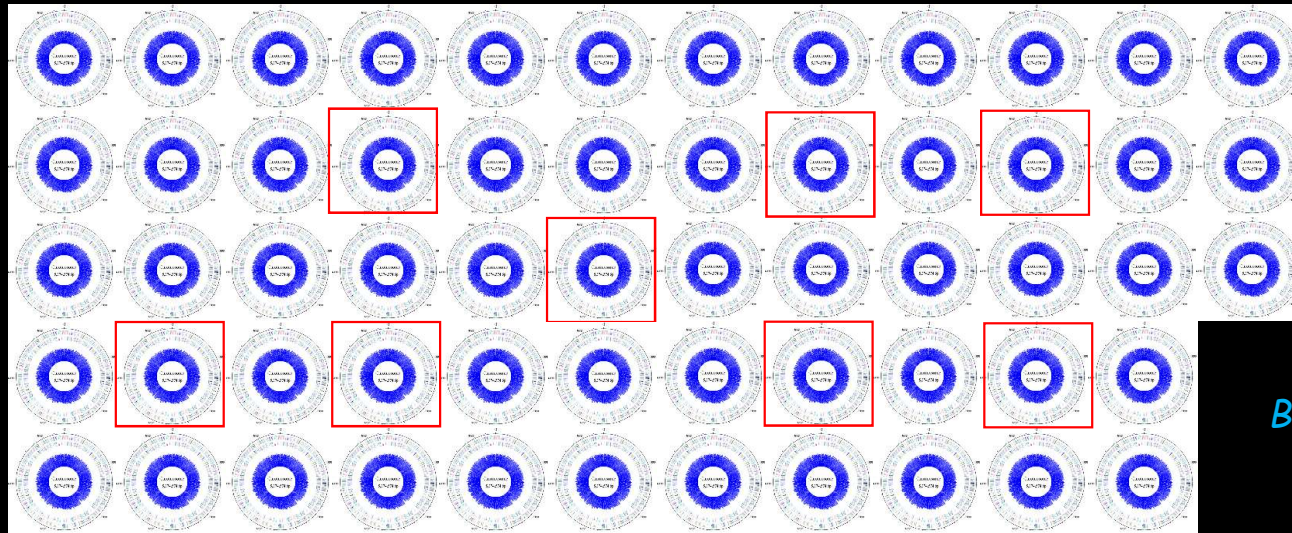
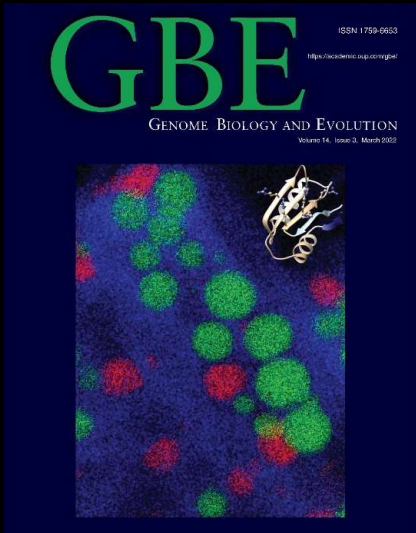
Optical microscopy  
@EPIDOR



→ Important Ca reservoir?  
→ What is the biological role of ACC?

*Gaetan et al. (2023) Env. Microbiol*

# Comparative genomics of iACC<sup>-</sup> vs iACC<sup>+</sup> cyanobacteria



Benzerara et al. (2022) GBE

⇒ One single "gene" shared by iACC<sup>+</sup> and absent in iACC<sup>-</sup>

iACC<sup>+</sup> cyanobacteria

*Gloeomargarita lithophora Alchichica-D10*  
*Synechococcus\_sp.\_PCC\_6312*  
*Microcystis aeruginosa\_PCC\_9443*  
*Fischerella\_sp.\_NIES-4106*

-6	-5	-4★	-3	-2	-1	0★	1	2	3	4	5	6																																										
T	I	G	G	M	V	G	L	M	G	H	V	V	G	S	A	I	G	G	V	A	G	A	V	T	L	G	P	V	G	A	V	I	G	A	Q	V	G	G	F	T	G	R	V	M	G	A	Q	L	G	E	T	L		
D	I	G	S	F	I	V	G	G	O	V	G	D	V	V	G	G	I	A	G	A	A	V	G	G	A	T	I	G	P	A	G	V	V	L	G	T	O	V	G	T	F	V	G	G	V	I	G	A	R	V	C	V	E	S
A	V	G	G	A	F	L	G	M	S	A	G	Q	V	A	G	G	M	I	G	G	A	I	G	S	.	A	L	G	P	G	G	A	V	L	G	A	Q	V	G	A	F	A	G	S	T	T	G	A	K	L	G	Y	D	V
E	F	G	G	S	L	I	G	A	T	V	G	E	T	V	G	G	T	L	G	A	V	V	A	G	I	A	M	G	P	A	G	A	I	V	G	T	O	V	G	S	V	V	G	F	T	I	G	A	Q	L	G	E	D	A

GlyZip1

Alignment of aminoacids

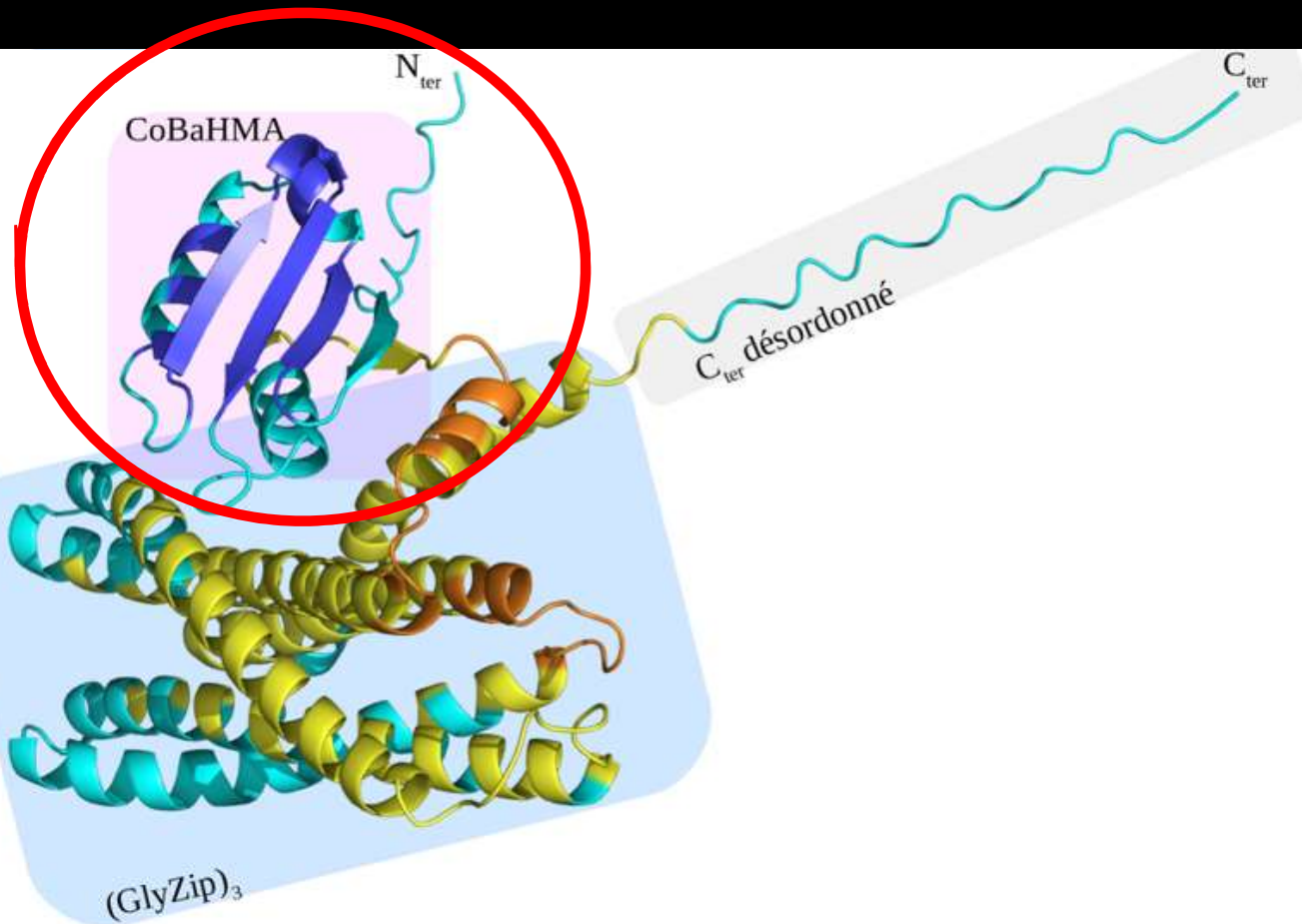


★ No detectable full-length homologs with known function in databases

⇒ New family of genes (*ccyA*)/proteins (calcyanin)



## Determining the structure of calcyanin

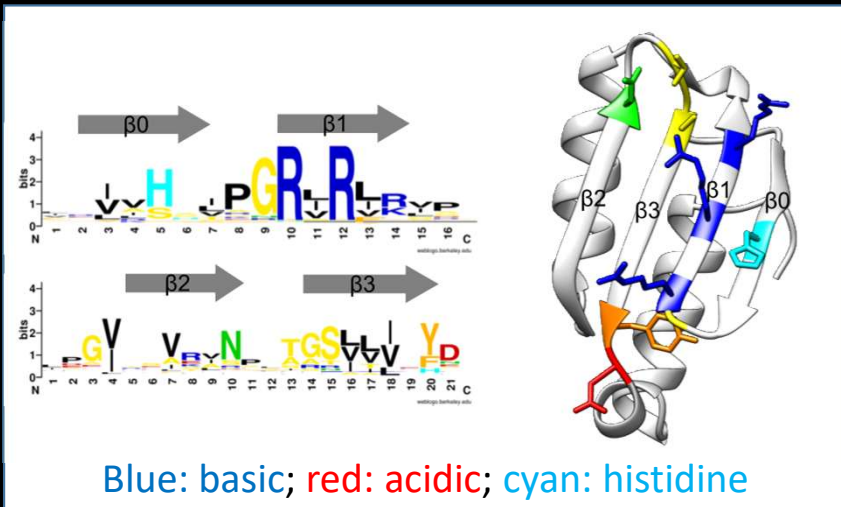


Alphafold2 (AI-based) prediction of the calcyanin of *Cyanothece* sp. PCC 7425

What is the role of these different modules (domains) in iACC formation?  
What do we know about the CoBaHMA domain?

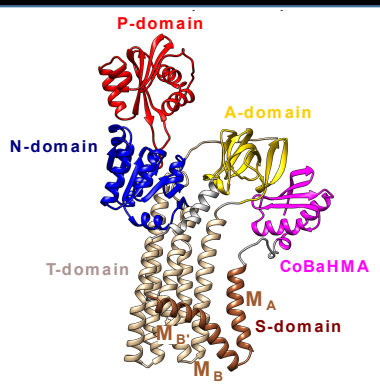
# What is CoBaHMA ?

Gaschignard et al. (2024) *Proteins*

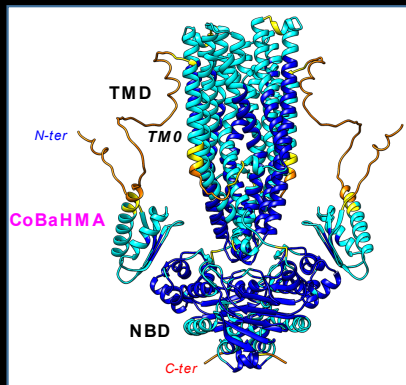


CoBaHMA found in 2227 different bacterial proteins  
>74 different types of protein modular organizations

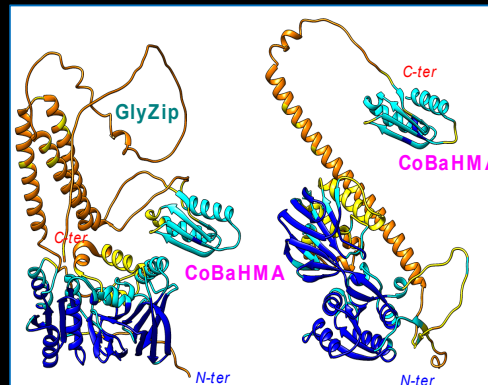
Membrane proteins involved in transport or lipid metabolism



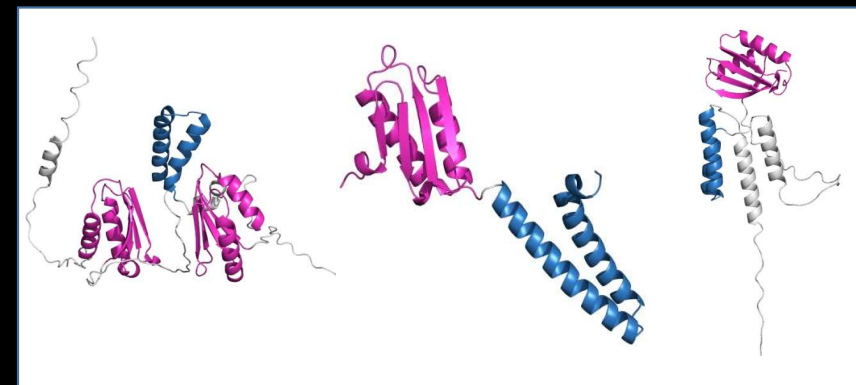
P1B-ATPases



ABC exporter



Diacylglycerol kinase

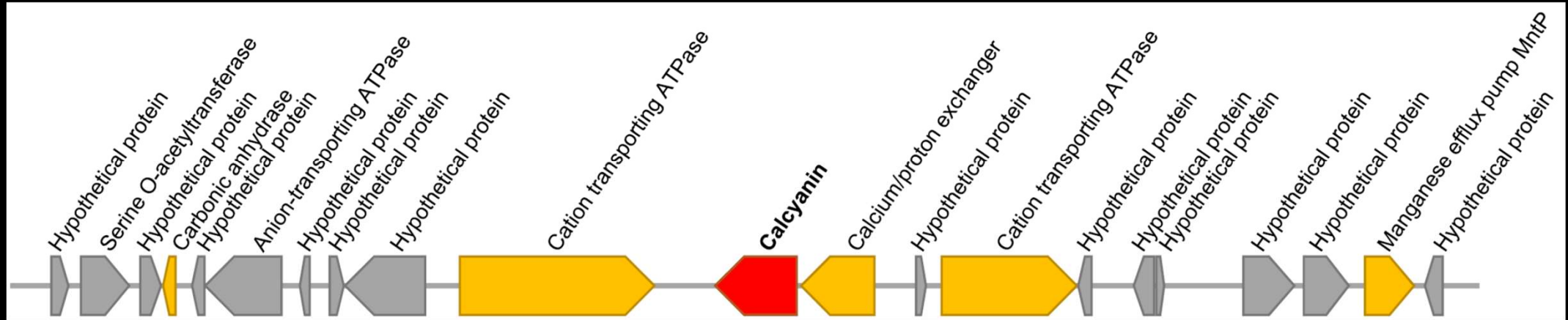


Unknown proteins with CoBaHMA+hairpins

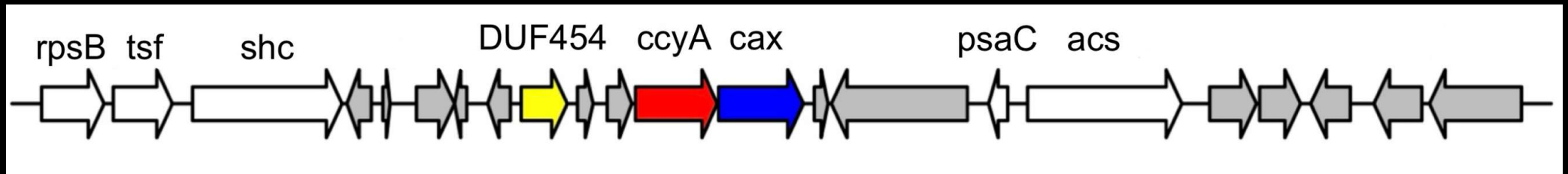
Regulatory function, involving interactions with anionic lipids ?

## Genomic neighboring of *ccyA*

→ *ccyA* in a megaplasmid in *Fischerella* sp NIES-4106.



→ *ccyA* in the genome of *Microcystis aeruginosa* PCC 7806



Some neighboring genes particularly interesting :

- ★  $\text{Ca}^{2+}/\text{H}^{+}$  exchanger (*cax*)
- ★ Domain of unknown function (DUF) encodes a CoBaHMA domain (no CoBaHMA in *Microcystis calcyanin*)



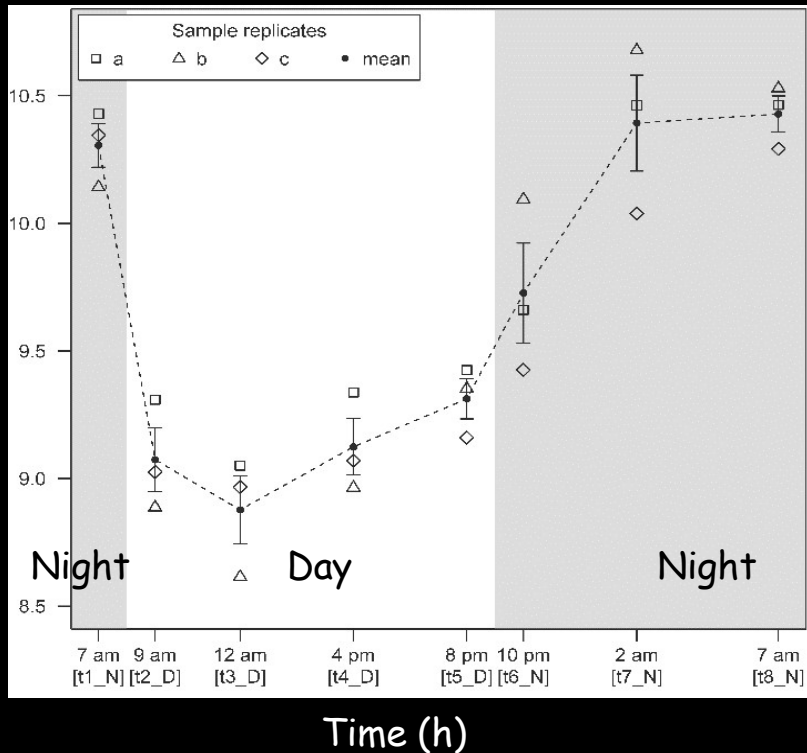
# Day/night expression of the *ccyA* genes



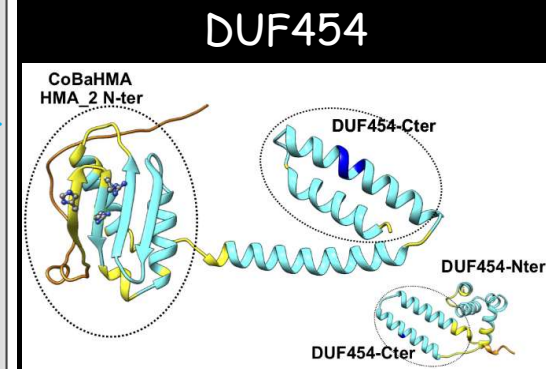
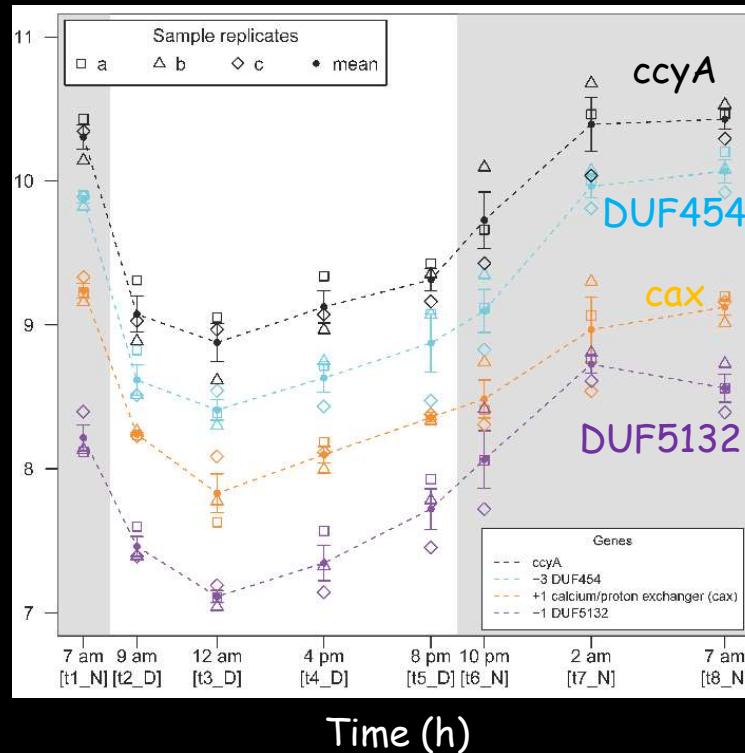
Abundance?

→ RNAseq experiments

Normalized expression of the *ccyA* gene



Normalized expression of the *ccyA* gene



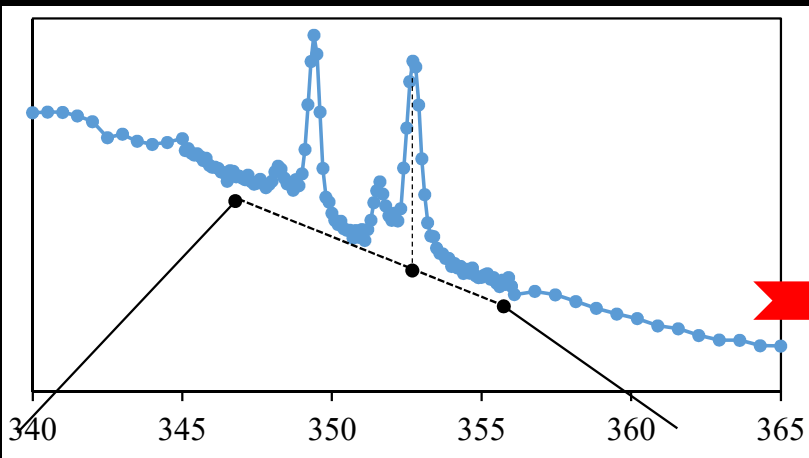
Expression of *ccyA* varies over a day/night cycle with higher expression at the end of the night

Expression of neighboring genes is correlated with that of *ccyA*: transcription unit?

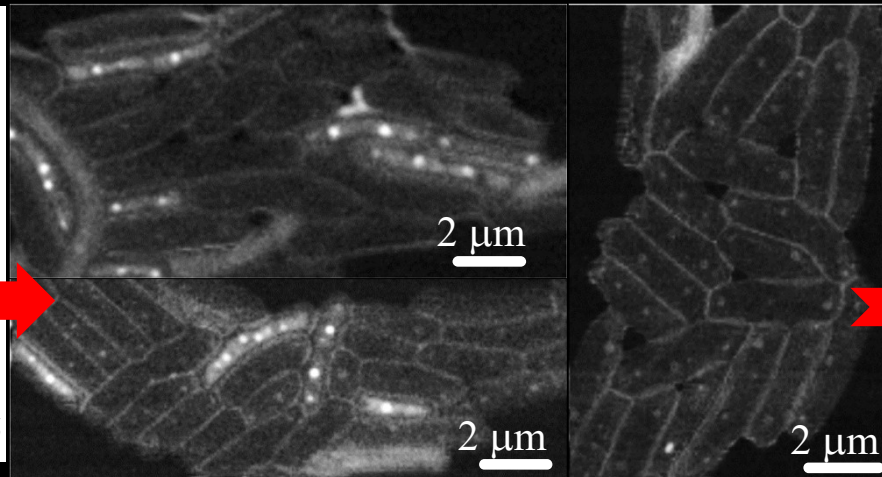
# Genetics: Expression of *ccyA* in a cyanobacterium not forming ACC

*Synechococcus elongatus* PCC 7942, wild type and mutants expressing *ccyA*

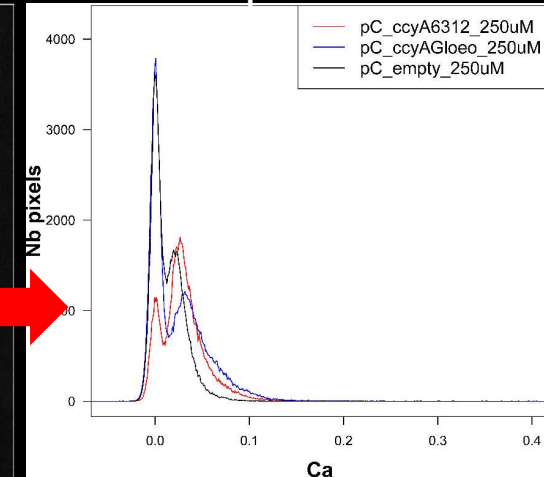
(Synchrotron) STXM at the Ca  $L_{2,3}$  edges



Ca maps (30 nm resolution)



Quantitative comparisons



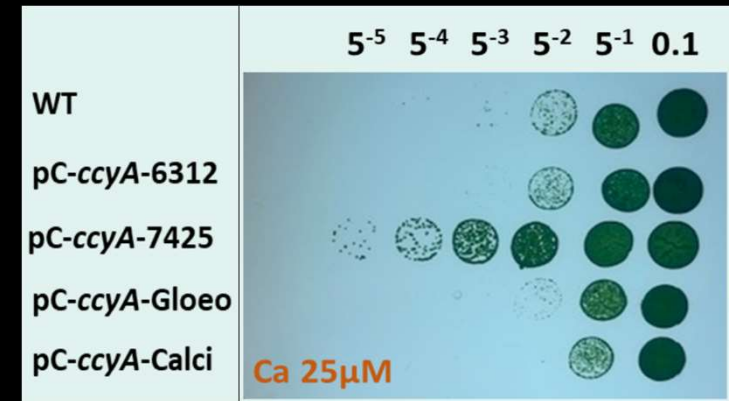
*Benzerara et al. (2023) Journal of Electron Spectroscopy and Related Phenomena*

Mutants expressing *ccyA* have higher Ca contents  
They do not form ACC

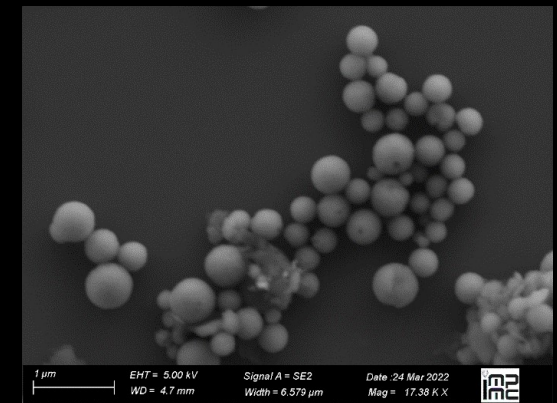
Need more than one gene to form intracellular ACC

## Ongoing work

→ Genetics and design of deletion mutants



→ iACC extraction and proteomics



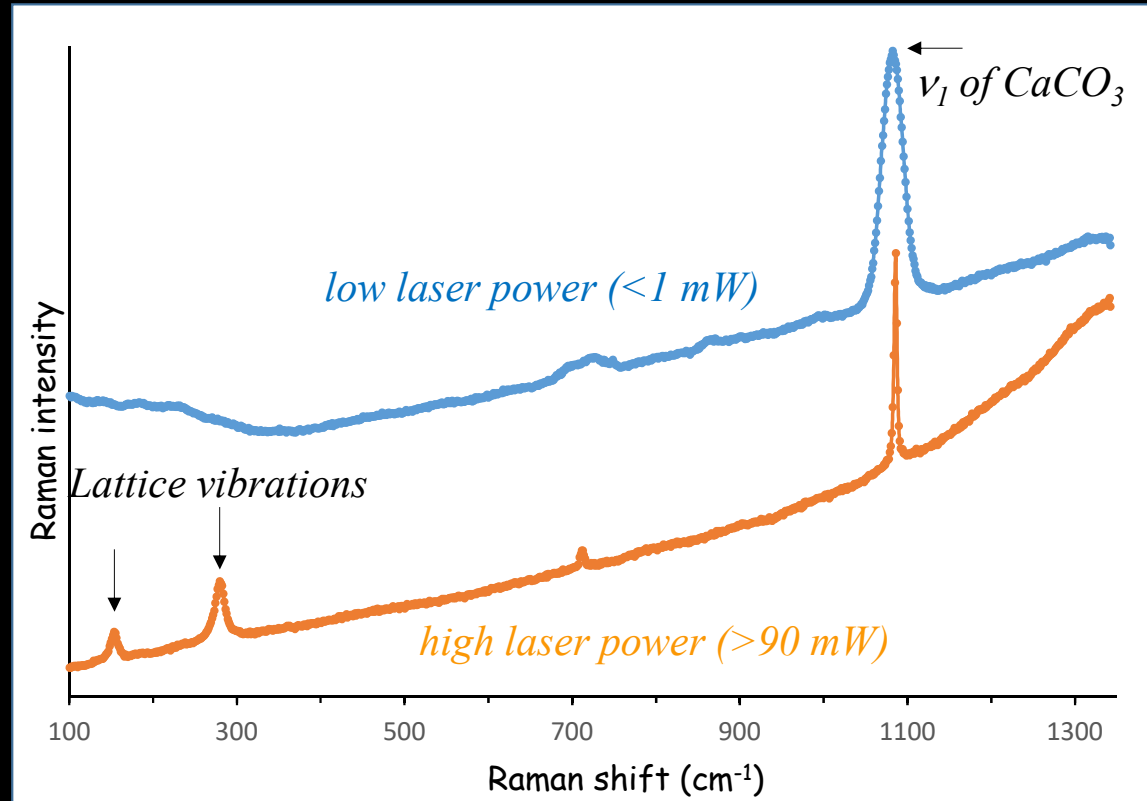
...

# Are cyanobacteria the only ACC-forming bacteria? NO

*Achromatium oxaliferum* (gammaproteobacterium)



Schewiakoff (1893)



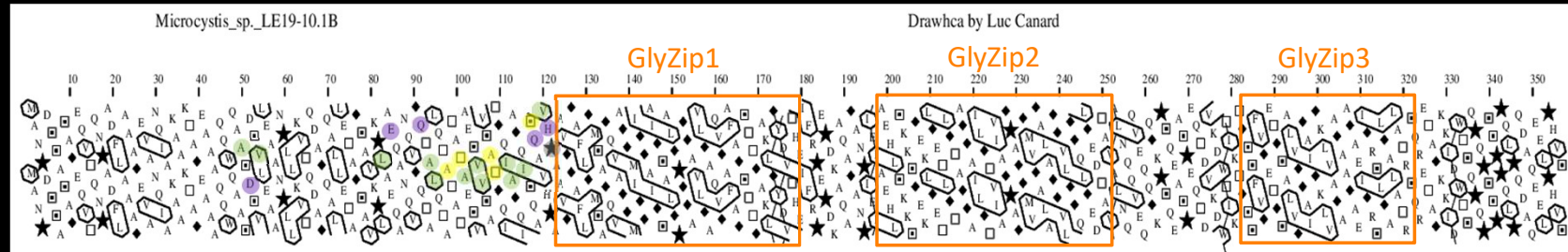
Benzerara et al. (2020) *Geobiol.*

Sulfoxidizing bacterium, massive amorphous carbonates

# The biomineralization of iACC in one gammaproteobacterium may be related to Cyanobacteria = *Methylococcus geothermalis*

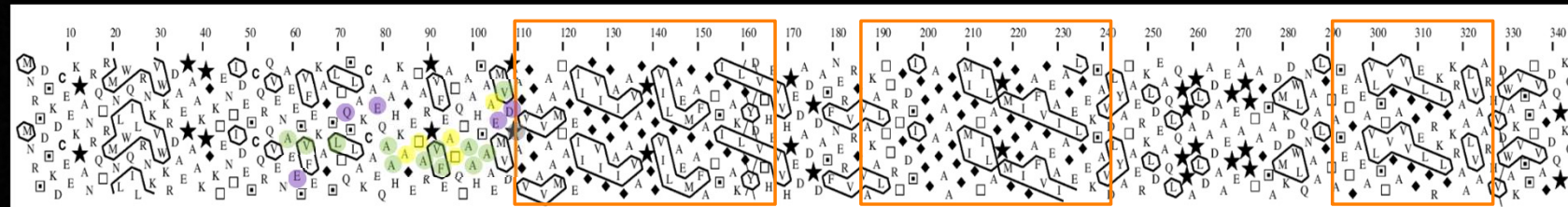
Calcyanin of a *Microcystis*

500 nm



Calcyanin of a *Methylococcaceae*

500 nm



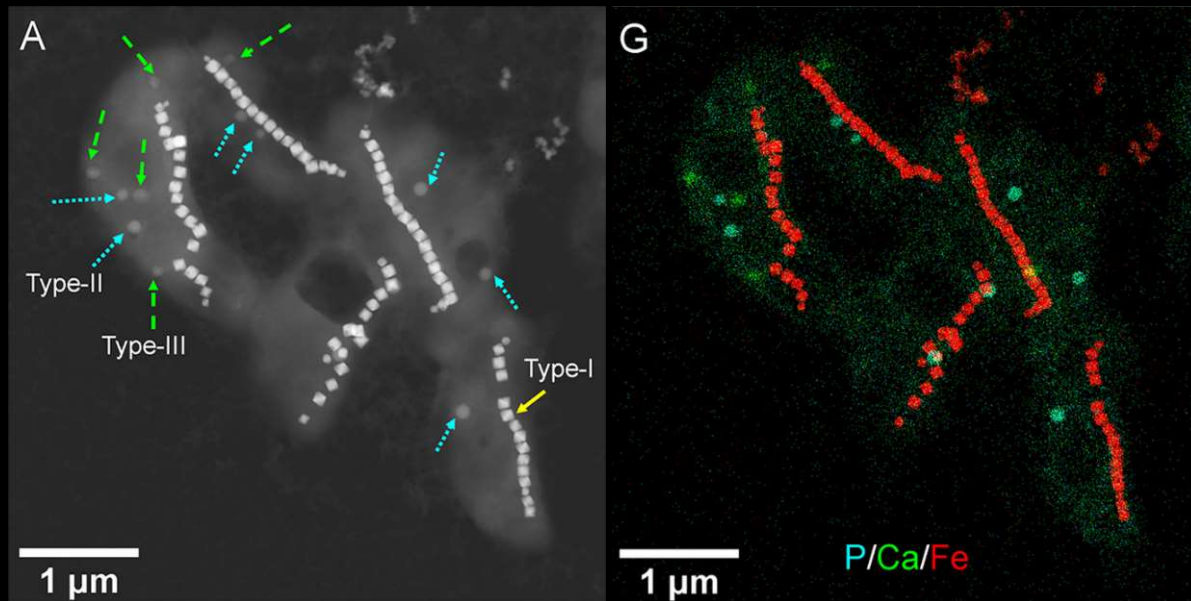
Benzerara et al., in prep

We find the *ccyA* gene in *Methylococcaceae*. Close to the one of *Microcystis*  
→ Horizontal transfer?



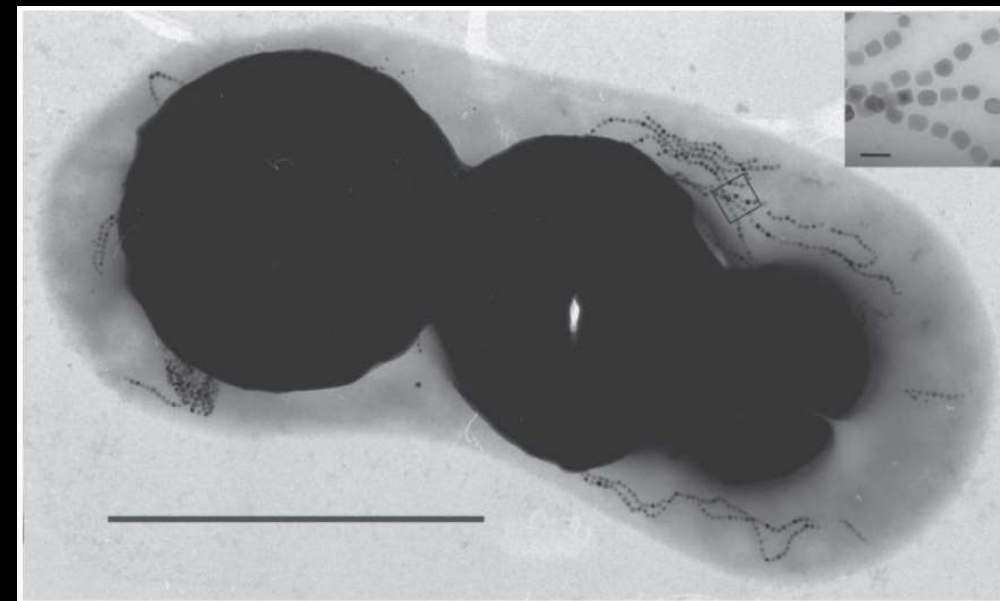
# Cosmopolitanism and diversity of MTB forming ACC

In Xingqingong Lake, Xi'an City



Alphaproteobacteria, Rhodospirillaceae (XQGS1),  
*Liu et al. (2021) AEM*

Freshwater pond in Kanazawa, Japan

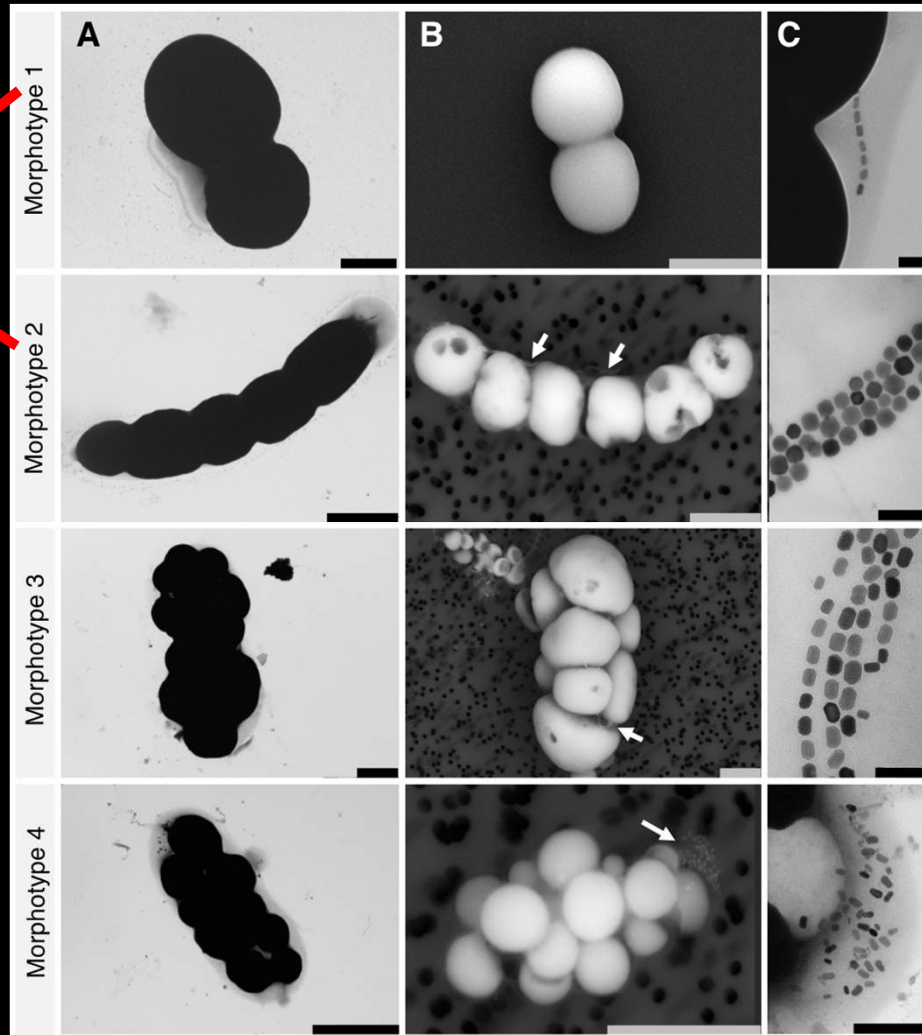
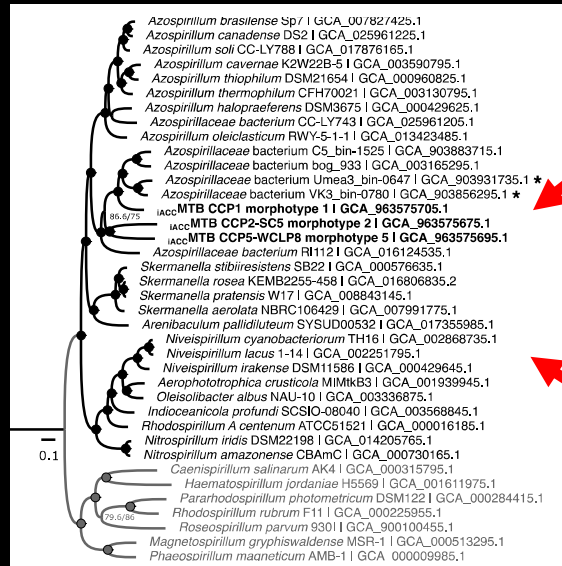


Gammaproteobacteria, Candidatus order/family  
CAIRSR01  
*Taoka et al. (2014) Microbiology*

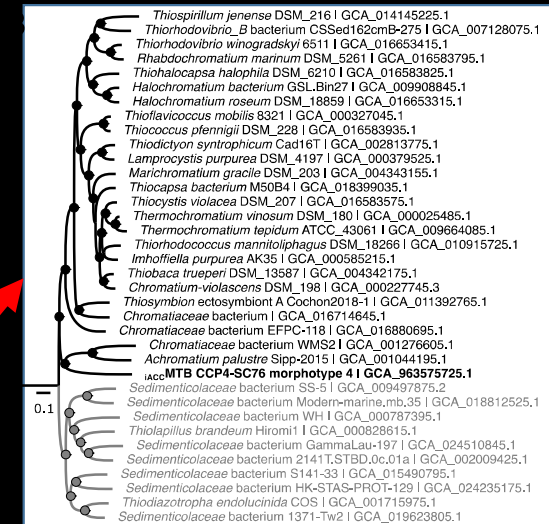
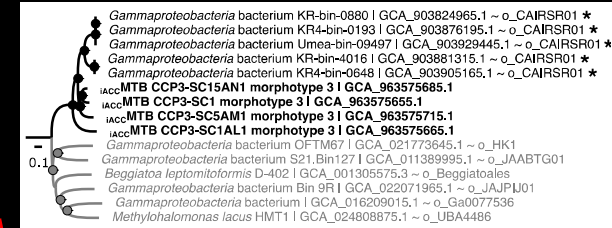


# Cosmopolitanism and diversity of MTB forming ACC

In Lake Pavin, Lake Bois le Roi, etc...



## Gammaproteobacteria, Candidatus order/family CAIRSR01

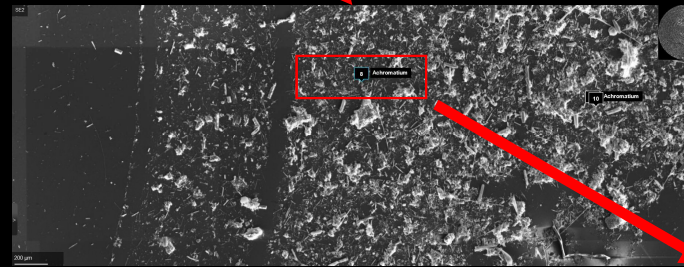
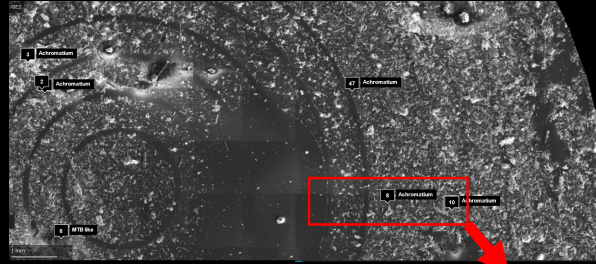
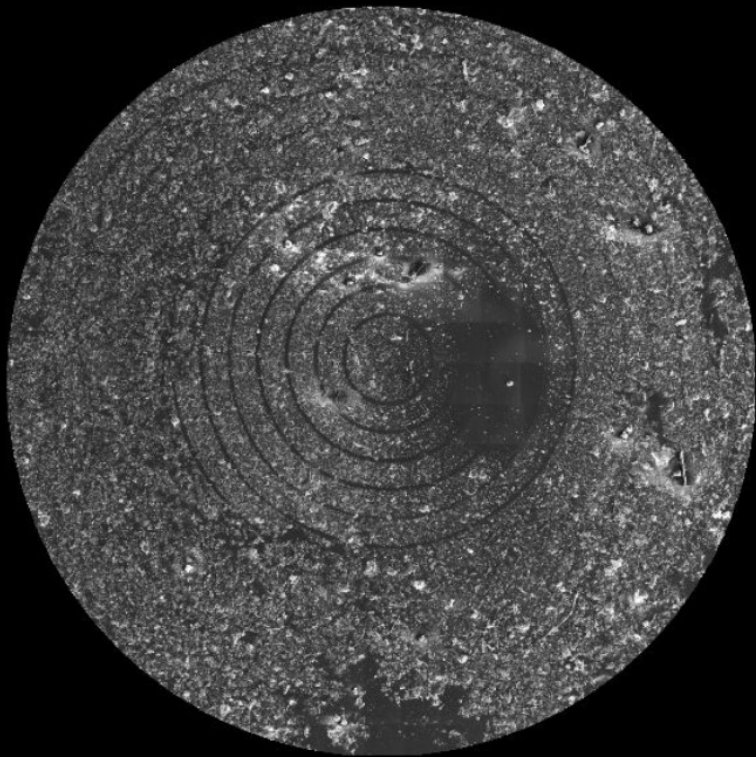


## Gammaproteobacteria, Chromatiaceae

Monteil et al (2020) ISME J; Mangin et al., in press

# ACC-forming MTB are abundant in Lake Pavin

Large SEM mosaics (5.9 Gpixels over ~4,3 cm<sup>2</sup>)

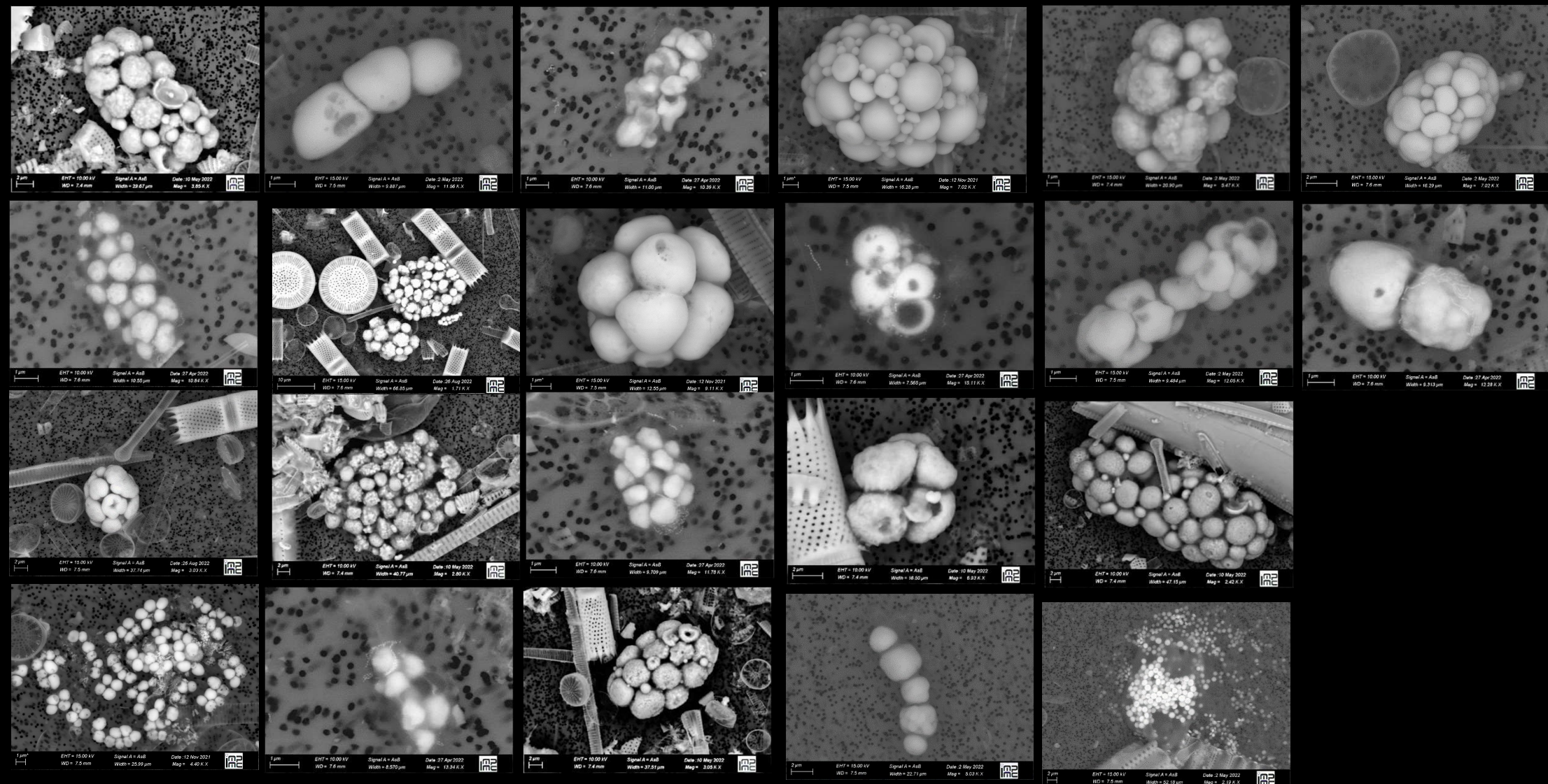


ACC-forming  
MTB





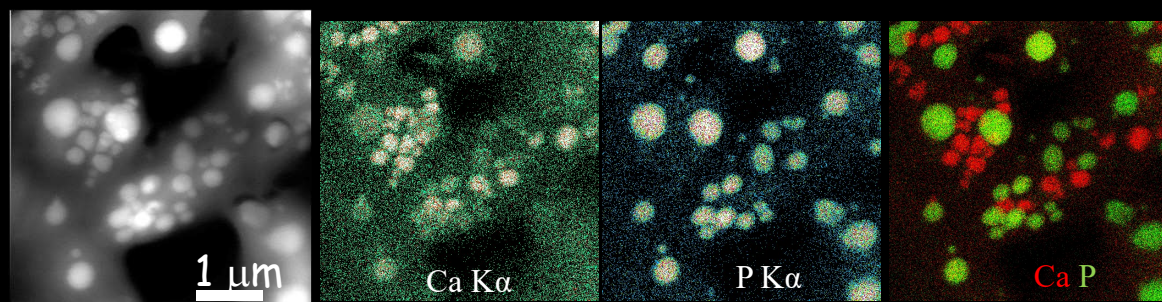
Tens to hundreds of cells per sample among millions (?) of particles. Need to detect them at different depths



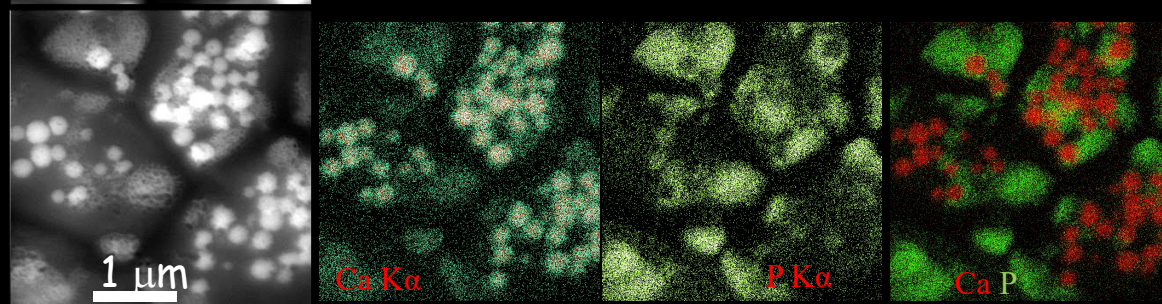
Ongoing development of a method to detect all of them efficiently, count them and assess their diversity



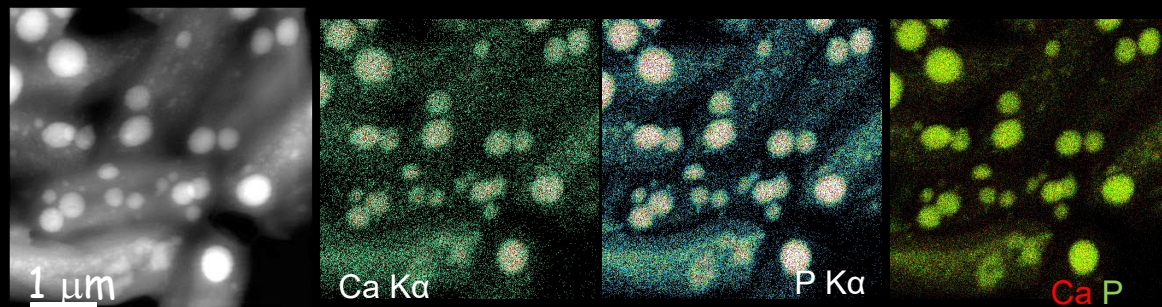
## Why have they been overlooked before?



Before cell  
fixation  
(glutaraldehyde)



After  
cell  
fixation



After  
dehydration

*Li et al. (2016) Minerals*

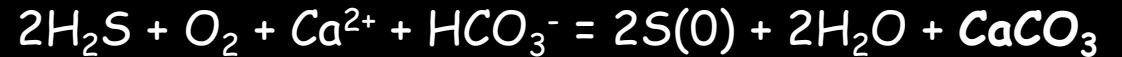
- ACC are lost upon classical sample preparation techniques
- Different response to magnetic field for ACC-forming MTB ([Monteil et al. 2020](#))

## Link between biomineralization and primary metabolisms?

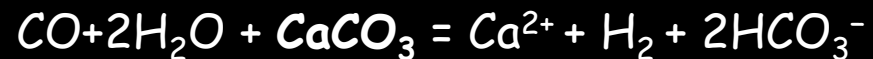
Oxygenic photosynthesis



Sulfoxidation



Aerobic methane  
oxidation/methylotrophy/carboxydotrophy



Some should favor ACC precipitation; other should favor dissolution

All seem to be able to fix  $\text{CO}_2$

## Shared origin among all bacteria?

- No certainty as long as we do not know how exactly bacteria form ACC
- If primary metabolism is part of the process, then it works differently for the different ACC-forming bacteria
- If *ccyA* is involved in cyanobacterial biomineralization, it is not found in MTB (unless we missed a remote homolog) but found in *Methylococcus*
- Lipid-bilayer compartment in MTB and *Achromatium*/ proteins in Cyanobacteriota
- Ca transport: vacuolar-type pumps in *Achromatium* and MTB (?) vs  $\text{Ca}^{2+}/\text{H}^{+}$  exchanger in cyanobacteria + other proteins

To be continued...



## (Causal role) Function(s)?

*Cosmidis and Benzerara (2022) Comptes rendus Géoscience*

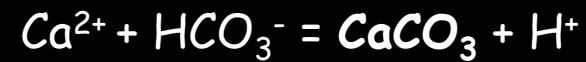
→ intracellular pH buffer/source of  $H^+$

→ Reservoir of inorganic C

→ Reservoir of Ca

→ Ballast

}  
}



ACC very reactive

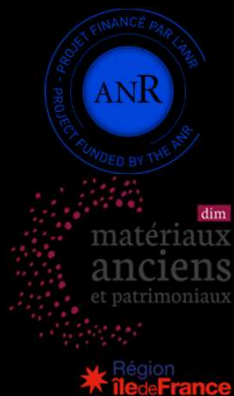
ACC density = 2.18 g/cm<sup>3</sup> (?)

Which parameter increased fitness so that this trait was eventually selected during evolution?

## Conclusions

- **Broad diversity** of bacteria able to biomineralize intracellular amorphous carbonates
- This diversity is **still most likely underestimated**, including within MTB
- This may have some **environmental impact**
- **Controlled mineralization** but molecular mechanisms and function remain **largely unknown**
- It most likely involves **more than one gene**. Some of these genes encode proteins/domains annotated as hypothetical or of unknown function
- **Evolutionary history?**

## Acknowledgements



ANR Harley, ANR-19-CE44-0017-01  
ANR Carbomagnet, ANR-21-CE01-0010-02  
ERC Calcyan, AFP7/2007-2013 Grant 307110



SEM, TEM, Spectroscopy facilities + GEMME @IMPMC; HERMES beamline @SOLEIL

**IMPMC** : Apolline Bruley, Isabelle Callebaut, Geraldine Caumes, Manuela Dezi, Elodie Duprat, Imène Esteve, Juliette Gaëtan, Geoffroy Gaschignard, Neha Mehta, Nicolas Menguy, Maxime Millet, Ferial Skouri-Panet, Cynthia Travert

**BIAM** (CEA Cadarache): Christopher Lefevre, Camille Mangin, Caroline Monteil, Daniel Chevrier, Catherine Berthomieu, Virginie Chapon

**IPGP**: Didier Jezequel, Eric Viollier

**I2BC** (CEA Saclay): Corinne Cassier-Chauvat, Franck Chauvat, Monis Khan

**Institut Pasteur**: Muriel Gugger

**ESE** (Univ Saclay): Purificacion Lopez-Garcia, David Moreira

**IEES**: Julie Leloup

**Key Laboratory of Earth and Planetary Physics, Beijing**: Jinhua Li