Spatially-resolved genomic, molecular organic, and stable isotopic analyses of an actively-accreting freshwater microbialite from Cuatro Ciénegas, Mexico

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Outline

• Ancient & Modern Microbialites
• Introduction to Cuatro Ciénegas
  – Conceptual approach & objectives
• Research Approach
  1. Community structure
  2. Molecular signatures
  3. Carbonate accretion
  4. Isotopic composition
• Summary: Integrated Conclusions
Ancient Stromatolites & Modern Microbialites
Ancient Stromatolites

Fossil Evidence of Early Life

• thought to be the product of some of the earliest biological communities on Earth (>3 billion years ago)
• abundant through much of the Proterozoic
• analogous to modern microbialites

Window to the past

• geochemical signatures are retained within the CaCO₃
• understanding the source of these signatures could help with the understanding of life processes on early Earth

Garcia-Pichel
Modern Microbialites

Living rocks
- organosedimentary microbial mats
- microbial consortia influences CaCO₃ accretion (trapping and precipitation)

Relatively uncommon
- found in diverse environments throughout the world
  - hot/cold, marine, freshwater, hypersaline
- environmental factors (water chemistry) are often conducive to CaCO₃ precipitation
Cuatro Ciénegas
Rio Mesquites

Spring-fed system
- Groundwater dependent habitat; karstic terrain
- Largest river in basin; 2 – 20 meters wide; 2.5 meters deep

Water Chemistry
- Rich in $\text{HCO}_3^-$, $\text{SO}_4^{2-}$
- Low $\text{Na}^+$, $\text{Cl}^-$
- Extremely high nitrate and low phosphate levels

Discharge
- Into man-made canals (flow out of basin)
- Formerly, into lagoons or “marshes” that gave the city of Cuatro Ciénergas its name

Living Microbialites
- Large amorphous and small round microbialites
An “organosedimentary” microbial mat

What makes a rock living?

As the microbial community traps sediment and produces excess organic material it migrates upwards and outwards.

Over time the surface becomes buried, and the physical, chemical and biological characteristics of the matrix change.
Bulk results show that a complex consortium of microorganisms, autotrophic and heterotrophic, aerobic and anaerobic, are associated with carbonate precipitation.

Metagenome has a high abundance of genes for:
• photosynthesis, all wavelengths
• diverse phosphorous cycling
• sulfo- & nitrogen-based lipids
• biofilm formation (colonization)
• motility, quorum sensing
• chemotaxis
• temporal regulation (circadian clock)
• extracellular polymeric substances (EPS)
Microscale analyses...
Beauty is in the details

- Gene presence ≠ gene expression/activity
- Need to shift from bulk analyses to detailed spatial & temporal analyses to microbially-mediated redox processes

Garcia-Pichel et al. 2004
Spatial structure and temporal regulation are critical for developing/maintaining chemical microenvironments and coordinating microbial processes.

“Detailed Dissection”

The beauty is in the detail.
Conceptual goal

detailed spatial analysis of the bacterial community and geochemical signatures in modern, actively-accreting microbialites to understand the processes by which they form

• the microbial community spans the surface of the microbialite

• sharp gradients in the chemical parameters that control CaCO$_3$ precipitation occur over mm scales

• this suggests that the organisms and processes responsible for CaCO$_3$ accretion vary across similar spatial scales
Isolation of microbialite layers

- these 5 layers span the upper 20 mm of the microbialite surface

Layer 1
Layer 2
Layer 3
Layer 4
Layer 5

1 m
Objectives

1. Characterize the bacterial communities of all 5 layers

2. Characterize the molecular composition of each layer to determine:
   a) if the lipid content directly reflects the observed microbial community
   b) how the biomass is degraded, preserved, or altered with depth

3. Determine the relative accretion of CaCO$_3$ at each layer to determine what organisms and processes contribute to microbialite formation

4. Characterize the carbon isotope composition of organic matter and CaCO$_3$ to help determine how different carbon cycling processes affect microbialite formation
1) Community Composition: A genomic analysis who’s there?
Methods: DNA extraction and amplification

Sample collection: Isolation of microbialite layers

Sample disruption & homogenization

DNA extraction

PCR amplification

Cloning & Sequencing

Quality and Dereplication - FastGroupII
- sequence length ≥ 300 nt
- group sequences with ≥ 97% similarity into species

Amplification of the bacterial 16S rRNA gene:
- for phylogenetic ID

Phylogenetic Identification – BLASTn
- comparison with GenBank database
**Results:** discrete bacterial communities

- 261 different species (399 total sequences)
- Little overlap in bacterial community of individual layers
- Validates layer-specific approach
Results: community composition
Layer 1

Proteobacteria

Deltaproteobacteria

Alphaproteobacteria

Cyanobacteria

Diatoms

*cyanobacteria dominated

n = 84

L1

L2

L3

L4

L5

Alphaproteobacteria

Cyanobacteria

Deltaproteobacteria

Uncultured

Proteobacteria

Eukaryote

Bacteroidetes
Heterotrophic metabolism dominates the interior

Phototrophic metabolism dominates the surface

Results

Layer 1

Layer 2

Layer 3

Layer 4

Layer 5

metabolic activities affect CaCO$_3$ solubility

photosynthesis

CO$_2$ uptake raises local pH; promotes CaCO$_3$ precipitation

sulfate-reduction

Ca$^{2+}$ + 2CH$_2$O + SO$_4^{2-}$ $\Rightarrow$ 2HCO$_3^-$ + H$_2$S + Ca$^{2+}$

$\Rightarrow$ CaCO$_3$ + H$_2$S + H$_2$O + CO$_2$
Conclusions: community composition

1) Discrete populations of bacteria in each layer

2) Photoautotrophic organisms dominate the surface
   - not observed at depth

3) Sulfate reducing $\delta$-proteobacteria are abundant at the interior
   - primarily layer 4

Question:
Does the lipid signature change in the same way as the genomic signature?
2) Biomarker Distribution: A molecular organic analysis
Why use lipid biomarkers?

• Like genetic signatures, certain lipid compounds are specific to a single group of organisms, making them “biological markers”

• Unlike genetic material, lipid compounds are often resistant towards decomposition processes, allowing them to be used to identify organisms long after they die
**Methods:** lipid extraction, separation, & ID

Sample disruption & homogenization →

Total Lipid Extraction →

Compound Class Separation →

Derivatization →

Compound ID and Quantification

- trying to detect compounds that match the genomic data

fatty acids

Accelerated Solvent Extraction (ASE) activated silica column

Gas Chromatography - Mass spectrometry (GC-MS)

- L1-alkanes_Total Ion Chromatogram
Results: changing signatures with depth

(C_{16:1}, n-7 + C_{18:1}, n-9)

Layer 1
Layer 2
Layer 3
Layer 4
Layer 5

Cyanobacterial biomarkers

Diatom biomarkers
**Results:** changing signatures with depth

**Layer 5: Two thiophene isomers**

- sulfurized derivatives of phytol

\[
\text{Phytol} + \text{H}_2\text{S} \Rightarrow
\]

- indicative of both the phototrophic community and sulfate reducing organisms
Conclusions: lipid composition

1) Lipid composition reflects community as determined by genomics:
   • abundant phototrophic biomarkers
   • Sulfate-reducing bacteria biomarkers

2) Photoautotrophic biomass is efficiently degraded by the heterotrophic community at depth

Question:
How does the distribution of organisms and changes in molecular composition relate to carbonate accretion?
3) Carbonate Accretion:
A mass balance analysis
Methods: microbialite composition

microbialite sample

dry sample

Carbon Coulometry

organic matter

carbonate

water

mass $H_2O$

mass $CaCO_3$

mass OM
Results: mass balance

- Low organic matter content
  - less than 6% (by wt)
  - decreases with depth

- CaCO$_3$ increases w/ depth
  - 35 – 90%
  - precipitation not only at surface

*Where does CaCO$_3$ precipitation occur?
Results: mass balance

Evolution of CaCO₃ Precipitation

• Increases in CaCO₃ reflect its accumulation from multiple generations of precipitation.
Conclusions: carbonate accretion

1) Carbonate accounts for the majority of the microbialite matrix
   • 90% in layer 5
   • low OM content throughout

1) Multiple generations of CaCO$_3$ precipitation
   • 2 distinct zones
   • directly adjacent to areas of high phototroph and sulfate-reducer abundance

Question:
Can the $\delta^{13}$C of CaCO$_3$ in the layers provide insight into what metabolic processes result in the precipitation of CaCO$_3$ in the different layers?
4) Carbon Cycling Processes: An isotopic analysis
Methods: carbon isotope analyses

- **dried microbialite sample**
  - Homogenize
  - Treat with 0.5 HCl

- **organic carbon**
  - Continuous flow IR-MS combustion analysis

- **inorganic carbon**
  - Homogenize
  - Analysis by dual-inlet IR-MS with Kiel

- **organic matter**
- **carbonate**
Results: carbon isotope profile

- δ\(^{13}\)C typical of cyanobacterial fractionation
- relatively \(^{13}\)C enriched
  - carbohydrate rich EPS
  - HCO\(_3\)\(^{-}\) utilization
- remineralization of \(^{13}\)C rich biomass (EPS?)
- CaCO\(_3\) δ\(^{13}\)C follows a similar trend to OM
- remineralization of OM
- δ\(^{13}\)C incorporates biogenic signature in CaCO\(_3\)

Organic Matter

Inorganic CaCO\(_3\)

δ\(^{13}\)C-DIC
+4 \(\%\)
Results: CaCO$_3$ $^{13}$C depletion

$\delta^{13}$C of individual CaCO$_3$ generation (calculated)
Conclusions: carbon isotope profile

1) Organic matter $\delta^{13}C$ reflects the community composition
   • photoautotrophs at surface
   • heterotrophic degradation at depth

1) The remineralization of OM by heterotrophic bacteria significantly affects the $\delta^{13}C$ of CaCO$_3$ at depth
   • incorporates a biological isotopic signature in the inorganic matrix
Summary:

integrated conclusions
Heterotrophic bacteria dominate the interior.

Photoautotrophic bacteria dominate the surface.

Summary

Layer 1

Layer 2

Layer 3

Layer 4

Layer 5

Cyanobacteria & Eukaryotic Algae

Proteobacteria & Heterotrophic Bacteria

Community composition

General bacterial distribution

Diagram showing layers with different bacterial groups.
Biomarkers support 16S
- numerous cyano & diatom lipids
- SRB lipids abundant
- $\delta^{13}C$ of organic matter

Signature changes w/ depth
- phototrophic markers lost
- sulfate reduction active at depth (if not dominant)
- shift from aerobic phototrophic to anaerobic heterotrophic system
Summary

<table>
<thead>
<tr>
<th>general bacterial distribution</th>
<th>dominant metabolic processes</th>
<th>CaCO$_3$ (% total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyanobacteria &amp; Eukaryotic Algae</td>
<td>oxic photosynthesis</td>
<td>0 20 40</td>
</tr>
<tr>
<td></td>
<td>aerobic respiration</td>
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<td></td>
<td>dysoxic anoxygenic photosynthesis</td>
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<td>Proteobacteria &amp; Heterotrophic Bacteria</td>
<td>anoxic anaerobic heterotrophy</td>
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<td></td>
<td>sulfate reduction</td>
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<td>Dominant Metabolic Processes</td>
<td>$\text{CaCO}_3$ (% Total)</td>
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<td>Dysoxic&lt;br&gt;Anoxygenic photosynthesis</td>
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<td></td>
<td>Anoxic&lt;br&gt;Anaerobic heterotrophy&lt;br&gt;Sulfate reduction</td>
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</table>

**Summary**

- **Oxic**: Photosynthesis, aerobic respiration
- **Dysoxic**: Anoxygenic photosynthesis
- **Anoxic**: Anaerobic heterotrophy, sulfate reduction
Conclusions

1. The visually distinct layers of the microbialite correspond to diverse consortiums of bacteria that are both taxonomically and physiologically different.

2. Various autotrophic and heterotrophic processes are associated with microbialite formation and carbonate precipitation, occurring at both the surface and interior.
   - photoautotrophic organisms dominate surface
   - heterotrophic organisms are more abundant at the interior

3. The observed alteration of geochemical signatures (both molecular organic and isotopic) from surface to the interior results in the preservation of a chemical “fingerprint” that can be used to interpret the total microbial community and the processes occurring within.
   - this fingerprint can also be applied to the interpretation of ancient systems
Implications: final molecular signature

A community fingerprint

- genetic material is quickly degraded – can not be exploited for analysis of ancient systems
- lipid components of the microbial biomass are preferentially preserved in the microbialite
Results: community composition

Cyanobacteria
  • Supports metagenomic work – 74% Cyano sequences (Breitbart et al. 2009)
  • Pleurocapsales similar to Highborne Cay, Bahamas clones

Diatoms
  • Detected by 16S; confirmed by 18S and SEM

α-proteobacteria
  • Group with purple non-sulfur bacteria species
  • Anoxygenic photoautotrophs

Bacteroidetes
  • Heterotrophic – exopolysaccharide degraders

Proteobacteria
  • 40 - 50% of clones in L3, L4, and L5
  • Diverse metabolic capacity
  • Sulfate reducing δ-proteobacteria
  • Anaerobic heterotrophy

L1
L2
L3
L4
L5
Results: notable lipid components

General bacterial lipids
  • hydrocarbons \(n\)-C\(_{16}\) through \(n\)-C\(_{22}\)
  • diploptene

Cyanobacterial lipids
  • unsaturated fatty acids
    - common in cyanobacteria and phototrophic eukaryotes
  • phytol
    - side-chain of chlorophyll-a & bacteriochlorophyll-a
  • mid-chain branched hydrocarbons
    - 18 – 20 carbons

Sulfate reducing bacteria
  • mid-chain methyl-branched fatty acids
  • sulfurized derivatives of phytol
    - indicative of both the phototrophic community and sulfate reducing organisms
Implications

The lipid content and $\delta^{13}$C-CaCO$_3$ values observed in layer 5 reveal some (not all) information about the composition and physiological function of the total community.

Can similar information be obtained from ancient fossilized stromatolites?
Microbialite metagenome shows a high degree of environmental adaptation

- Phosphate sensing and regulation (Pho regulon)
- Phosphate transporters (Pst genes)
  - Genes induced by P starvation (alkaline phosphatase) removes phosphate groups from nucleotides/ proteins
- Phosphonate utilization (C-P bond): general C-P lyase and specific phosphonatases
  - phosphonates may be prebiotic carriers of phosphorus
- Polyphosphate metabolism (polyphosphate kinase/
  - exopolyphosphatase)
  - polyphosphate strongly chelates metals (e.g., Ca) – may play role in carbonate precipitation
- Use of sulfolipids instead of phospholipids
Taxonomic Composition (~30,000 seqs)

- Cyanobacteria (74.4%)
  - Nostocales
  - Chroococcales
  - Oscillatoriales

- Archaea (0.7%)
  - Mostly euryarchaeota

- Mobile (0.3%)

- Other Heterotrophs (9.0%)

- Proteobacteria (11.2%)
Genomic evidence for Photosynthesis

- Cyanobacterial sequences were abundant (74%)
- Genes for both light-dependent and light-independent reactions
- Photosynthetic CO₂ uptake raises pH locally, which promotes CaCO₃ precipitation
- Genes for photosynthetic pigments to access a wide variety of light wavelengths
  - **Chlorophyll** absorbs blue & red light (430 and 660 nm)
  - **Phycoerythrin** absorbs green light (540-570 nm)
  - **Phycoerythrocyanin** absorbs yellow light (570 nm)
  - **Phycocyanin** absorbs yellow-orange light (620-655 nm)
  - **Allophycocyanin** absorbs red light (650 nm)
  - **Phytochromes** absorb near infra-red light (650-740 nm)
The microbialites contain a diverse community of autotrophic and heterotrophic microbes performing aerobic and anaerobic processes

How are their activities coordinated?

Metagenome has a high abundance of genes for:
- biofilm formation (widespread colonization island)
- motility
- chemotaxis
- quorum sensing
- temporal regulation (circadian clock genes)
- extracellular polymeric substances (EPS)
Extracellular Polymeric Substances (EPS)

EPS Genes in Metagenome

• EPS synthesis
  alginate, colonic acid, sialic acid, rhamnose-containing glycans
• EPS degradation
  sulfatases, hydrolases, glycosidases, lyases

Roles of EPS

• Aids in the development of sharp geochemical gradients and stable microenvironments
• Binds and concentrates calcium ions, inhibiting CaCO$_3$ precipitation
• Microbial degradation of EPS releases the calcium, favoring localized precipitation

Interestingly, some microbes produce maximum EPS in the absence of phosphate
Karst terrain in Jurassic & Cretaceous Platform Carbonates

**Laguna Churince**

**Poza Azules II**

**Poza Becerra**
MINERAL GENETIC PHENOMENA IN THE POOLS

Rising of hot sulphur waters rich in alkaline and alkaline-earth metals

Pozas Azules Microbialites

Bacteria biomass controlling sulphur oxidation to sulphates

aeolian deflation transports sand to the gypsum dunes

mixed sulphate crusts

Rising of hot sulphur waters rich in alkaline and alkaline-earth metals
In this karst terrain, the ponds and microbialites are ephemeral

- Cuatro Cienegas = 4 Marshes
- At the time of settlement the valley was filled with water
- Climate likely controlled ground water table in past
Poza Roja – An Extreme Environment

Very hot (~65°C) and salty

High pH (~12)

Microbial mats and layering
Poza Roja Sediments
A Microbial-Climate Archive?

- Salt crust with an exotic microbial consortium likely to include
  Purple sulfur-oxidizing bacteria
  Iron oxidizing bacteria
  Anaerobic photoautotrophs
  Other primitive life form

- Likely represent the influence that varying hydrologic conditions have on the water levels and chemistry in the pozas and the dominant microbial community.

- Strongly laminated with high frequency variations: A Climate Record? Need to evaluate climate history of region
MAN is NOT the only Threat to the Pozas and the Microbialites