**In Vivo Entombment of Bacteria and Fungi during Calcium Oxalate, Brushite and Struvite Urolithiasis**

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Abstract

Background: Human kidney stones form via repeated events of mineral precipitation, partial dissolution and reprecipitation, which are directly analogous to similar processes in other natural and man-made environments where resident microbiomes strongly influence biomineralization. High-resolution microscopy and high-fidelity metagenomic (microscopy-to-omics) analyses, applicable to all forms of biomineralization, have been applied to assemble definitive evidence of in vivo microbiome entombment during urolithiasis.

Methods: Stone fragments were collected from a randomly chosen cohort of 20 patients using standard percutaneous nephrolithotomy (PCNL). Fourier transform infrared (FTIR) spectroscopy indicated that 18 of these patients were calcium oxalate (CaOx) stone formers, while one patient each formed brushite and struvite stones. This apportionment is consistent with global stone mineralogy distributions. Stone fragments from 7 of these 20 patients (5 CaOx, 1 brushite and 1 struvite) were thin sectioned and analyzed using brightfield (BF), polarization (POL), confocal, superresolution autofluorescence (SRAF) and Raman techniques. DNA from remaining fragments, grouped according to each of the 20 patients, were analyzed with amplicon sequencing of 16S rRNA gene sequences (V1-V3, V3-V5) and internal transcribed spacer (ITS1, ITS2) regions.

Results: Bulk entombed DNA was sequenced from stone fragments in 11 of the 18 CaOx patients, as well as the brushite and struvite patients. These analyses confirmed the presence of an entombed low-diversity community of bacteria and fungi, including Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria, and Aspergillus niger. Bacterial cells ~1 μm in diameter were also optically observed to be entombed and well-
preserved in amorphous hydroxyapatite spherules and fans of needle-like crystals of brushite and struvite.

**Conclusions:** These results indicate a microbiome is entombed during *in vivo* CaOx stone formation. Similar processes are implied for brushite and struvite stones. This evidence lays the groundwork for future *in vitro* and *in vivo* experimentation to determine how the microbiome may actively and/or passively influence kidney stone biomineralization.

**Introduction**

Calcium-rich human kidney stones composed of the minerals calcium oxalate (CaOx, CaC$_2$O$_4$), calcium phosphate (hydroxyapatite Ca$_{10}$(PO$_4$)$_6$(OH)$_2$, and brushite (CaHPO$_4$)) are the most common products globally of urolithiasis in industrialized nations (1). Integrated approaches from geology, biology and medicine to study universal biomineralization in humans, animals and plants, called GeoBioMed, has recently shown that these kidney stones are formed via repeated events of precipitation, partial dissolution and reprecipitation (2, 3). Each of these biomineralization steps is promoted and/or inhibited by a variety of physical, chemical and biological processes and mechanisms (4). Importantly, the deposition of minerals that form human kidney stones (5) is directly comparable to biomineralization processes in other natural and man-made environments (6), all of which are strongly influenced by microorganisms (collectively called the microbiome) (7). For example, in hot-spring calcium carbonate (CaCO$_3$) travertine deposits, bacteria control mineral growth rate, mineralogy, and crystalline structure (6). Additionally, fungi excrete a number of organic acids (e.g. citric, oxalic and formic acids, among many others) that drive limestone dissolution and reprecipitation in
both natural and man-made settings (8). Similar types of microbe-urine-mineral interactions are thought to influence human kidney stone formation (5). For instance, previous microbial culturing and 16S rRNA gene sequencing from human kidney stones have detected both bacteria and fungi (9-12). As another example, oxalate-metabolizing bacterial networks in the gut microbiome have also been identified as a contributing factor to hyperoxaluria and the simultaneous formation of CaOx kidney stones (13). Furthermore, struvite stones have been shown to be influenced by the metabolic activity of urease-producing bacteria (14).

Given this background, the present study has integrated optical microscopy with metagenomic analyses (herein called microscopy-to-omics) to collect definitive direct evidence of in vivo microbiome diversity, entombment and preservation during human kidney stone formation. This project was undertaken to analyze stone fragments collected from a randomly chosen cohort of 20 patients using standard percutaneous nephrolithotomy (PCNL). This experimental design requires that analytical results from all 20 patients be presented, which included 18 calcium oxalate (CaOx) stone formers, one brushite stone former and one struvite stone former. The relatively large number of CaOx patients has permitted conclusive interpretations to be drawn regarding in vivo microbiome diversity, entombment and preservation. In contrast, the small number of brushite and struvite stone forming patients prevents similarly conclusive characterizations. However, their microscopy-to-omics analyses are nonetheless extremely valuable and serve as a useful pilot study comparison with previously published microbiome analyses of CaOx, brushite and struvite kidney stone fragments. Similar applications of microscopy-to-omics analyses, aided by comparisons with biomineralization in other natural and manmade environments, can be used to design
future in vitro and in vivo experimentation dedicated to finding new therapeutic interventions for the prevention and treatment of urolithiasis.

**Materials and Methods**

The methods used in this study are briefly summarized here and presented in detail in the Supplementary Materials. This includes a flow chart of the systematic analyses applied in this study (Figure 1). Kidney stone fragments were collected from a cohort of 20 randomly chosen Mayo Clinic patients (Supplementary Table 1). All patients received antibiotics for a minimum of 7 days before surgery. Medical history, standard serum labs, medication intake and comorbid conditions were assessed. In addition, 24-hour urine supersaturation profiles were obtained from all patients after surgery (Supplementary Table 2). To collect enough entombed microbial DNA, multiple stone fragments were grouped from each patient, washed in deionized water, air dried and analyzed for bulk mineralogical composition using Fourier Transform Infrared Spectroscopy (FTIR) at the Mayo Clinic Metals Laboratories (Supplementary Table 3). Subsets from seven of the patient-specific fragment groups (5 CaOx, 1 brushite, 1 struvite; Figure 1) were three-dimensionally oriented, impregnated with epoxy and made into 25 µm-thick, doubly-polished, uncovered thin section by Wagner Petrographic (Lindon, Utah) (3). Thin section microscopy analyses were done in the Microscopy Core of the Carl R. Woese Institute for Genomic Biology (IGB) on a Zeiss Axio Zoom.V16, a Zeiss Axio Observer Widefield System, a Zeiss LSM 880 Laser Scanning Microscope with Airyscan Superresolution, and a WITec Alpha 300RAS Raman System.

Another subset of each of the 20 patient-specific kidney stone fragment groups were collected for metagenomic analyses by flash freezing in the operating room immediately
after PCNL collection via placement in a -80°C Taylor-Wharton CX Series dry shipper dewar (Borehamwood, UK). The dewar was shipped overnight to the Illinois IGB where the fragment cohorts were stored at -80°C until analyzed. Thawed fragment groups were powdered with a sterilized mortar and pestle under a sterile laminar flow hood. Molecular sequencing and bioinformatic analysis of the V1-V3 and V3-V5 hypervariable regions of bacterial 16S rRNA gene sequences, human host non-ribosomal DNA fragments and fungal internal transcribed spacer (ITS) regions (ITS1 and ITS2) were conducted in the Illinois Roy J. Carver Biotechnology Center. The 16S rRNA gene sequences and ITS regions were analyzed on a Fluidigm™ system. Paired-end sequencing was completed on an Illumina™ MiSeq platform. Amplicon sequence variants (ASVs) in control samples were identified as contaminants and removed. Phylogenetic diversity analysis and statistical analyses were completed using the Phyloseq v1.22.3 (15) and R programs (16). Statistical correlations were assessed using Wilcoxon signed-rank and Fisher’s exact test.

**Ethics Approval and Consent to Participate**

This basic medical research study was reviewed and approved by the Institutional Review Board (IRB 09-002083) at the Mayo Clinic. Written informed consent was obtained from all patient participants and are on file with the Mayo Clinic in Rochester, Minnesota.

**Availability of Data and Materials**

The raw metagenomic sequencing data and other raw images and finalized images could be retrieved from the following link.

https://uofi.box.com/s/czaik83my8srldz09qd3mhp7zygn3s87
Kidney stone fragment thin sections and all data curation is available from M.S.

Results

Entombment of bacterial, fungal, and human host amplicon sequences

The V1-V3 and V3-V5 hypervariable regions of bacterial 16S rRNA gene sequences and human host non-ribosomal DNA fragments were detected in 9 out of 20 patient-specific kidney stone fragment groups (7 CaOx, 1 brushite, 1 struvite; Figures 1, 2a). From these, a total of 214 unique ASVs were identified (Figure 2a, Table S4). Host human non-ribosomal DNA sequences were dominant in four of the seven CaOx fragment groups (Figure 3a, Figure S1). In the brushite stone fragment group, 99.5% of the total sequence reads were classified as host human non-ribosomal DNA sequences (Figure 3c). Conversely, no host human non-ribosomal DNA was detected in the struvite fragment group (Figure 3e). The 40 unique ASVs identified in the struvite fragment group were slightly more diverse than those detected in the CaOx and brushite fragment groups (Shannon Index = 0.84, Simpson Index = 0.35). The struvite fragment group bacterial community composition was dominated by *Staphylococcus* (79.8% of reads, Figure 3e) and included *Porphyromonas* (6.5%), *Abiotrophia* (5.7%), and *Haemophilus* (1.7%). Fungal amplicon sequences were detected in 11 out of 20 fragment groups (9 CaOx, 1 brushite; 1 struvite; Figures 1, 2b). ASVs attained from all stone fragment groups were predominantly *Aspergillus niger*, with the remaining 36 ASVs affiliated with *Aspergillus*, Basidiomycota, and Agaricomycetes. In addition, *Aspergillus nomius*, *Aspergillus costaricensis*, *Candida albicans*, *Candida dubliniensis*, and *Dothideaceae* (family-level) constituted <1% of the total community (Figure 3b, d, f).

Microbiome partitioning between stone mineralogy types
The 18 patients that formed CaOx kidney stone fragment groups permit firm conclusions to be drawn with respect to \textit{in vivo} microbiome diversity, entombment and preservation. However, the single brushite and struvite fragment groups permit only pilot study-level initial comparison with the CaOx fragment groups. The CaOx, brushite, and struvite fragment groups each contain significantly different entombed bacterial communities (Figure 2c). Bacteria in the brushite fragment group share only one taxa (\textit{Staphylococcus}) with the CaOx and struvite fragment groups. Additionally, host non-ribosomal DNA was identified in the CaOx and brushite fragment groups, but not in the struvite fragment group (Figure 2c). Fungal communities exhibited more overlap amongst the three CaOx, brushite and struvite fragment group mineralogy types (Figure 2c). \textit{A. niger} dominated the fungal communities of all three fragment mineralogy types, while \textit{A. nomius} constituted less than 1\% of these fungal communities. CaOx and struvite fragment groups shared \textit{Aspergillus}, while the CaOx and brushite fragment groups shared \textit{Agaricomycetes}.

\textbf{Correlation of fungal microbiome with urinary calcium and oxalate excretion}

The 11 patient-specific kidney stone fragment groups containing fungal sequences do not exhibit statistically significantly correlations with higher patient urine calcium excretion at the $\alpha = 0.05$ level (Figure 4a; $p = 0.07$). However, mean calcium concentrations in 24-hour urine analyses are higher in the presence of fungal sequences (“absent” mean = 180.3 mg/24-hr; “present” mean = 308.8 mg/24-hr; Figure 4a). Similarly, there were no statistically significant correlations observed between presence and absence of fungal sequences and urine oxalate concentrations (Figure 4b; $p = 0.15$). However, patients with fungal sequences in their stone fragment groups exhibited lower mean urine oxalate concentrations (20.2 mg/24-hr) than patients without
fungal sequences (33.0 mg/24-hr). Patient-specific fragment groups with fungal sequences also exhibited statistically insignificant (p = 0.33) increases in average 24-hour urine CaOx saturation indices (1.6 DG/24-hr without; 2.0 DG/24-hr with). Additionally, there was no statistically significant correlation between the presence or absence of fungal sequences and patient 24-hour urine citrate levels (p = 0.4). Finally, the presence or absence of apatite in each patient-specific fragment group correlates with the presence of fungal sequences (p = 0.02; odds ratio = 0.05).

Microbiome entombment within amorphous and crystalline minerals

Microscopy was completed on five CaOx, one brushite and one struvite patient-specific kidney stone fragments. While bacteria and fungi were detected in the metagenomic analyses for the CaOx stone fragments (Figure 3), neither entombed bacterial cells or fungal hyphae borings were observed with microscopy. It is important to note that metagenomic analyses result from a significantly higher volume coverage of each of the patient-specific stone fragment groups and are therefore expected to yield more evidence of an entombed microbiome than the small 25 μm-thick cross-sectional area investigated by thin section microscopy analyses. Conversely, brushite and struvite stone fragments contained both bacterial and fungal metagenomic sequences (Figure 3). While they clearly exhibited entombed bacterial cells within their amorphous spherulitic hydroxyapatite (Figures 5-8), no fungal hyphae were observed. In the struvite stone fragment, concentrically-layered amorphous hydroxyapatite spherules and radiating needle-like (acicular) struvite crystals contain entombed coccoid- and rod-shaped bacteria ~1 μm in diameter (Figures 5 and 6). Similarly, the brushite fragment contained entombed bacteria within concentrically-layered amorphous hydroxyapatite
spherules and radiating acicular brushite crystals (Figures 6e and f, 8). In addition, Raman spectroscopy has identified entombed organic biomolecules including lipids and proteins and confirmed that amorphous spherules are comprised of hydroxyapatite (Figure 7).

Discussion

Phylogenetic Diversity of the Entombed In Vivo Microbiome

The microscopy-to-omics analyses assembled in the present study provide important microscopy and genomic evidence of entombment of a low-diversity community of microorganisms during in vivo CaOx stone formation of (Figures 2 and 3). Optical and genomic evidence in the present pilot study analyses of the brushite and struvite patient-specific kidney stone fragments show that similar processes are implied for brushite and struvite stones (Figures 2-8). The human microbiome, in combination with both genetic and environmental factors, has been implicated as playing an important role in urolithiasis (5, 17-19). This is supported by several previous studies using both culturing and sequencing techniques that have detected a microbiome within CaOx stones (10-12), brushite stones (12), struvite stones (14) and urine (12, 20).

*Staphylococcus* was detected in both the current study (Figure 3a) and previous analyses of CaOx stone fragments (10-12). However, no overlap was observed in bacterial diversity between the present pilot study (Figure 3c) and previous studies of brushite stone microbiology (12). Conversely, *Pseudomonas and Staphylococcus* were found in common between the present pilot study (Figure 3e) and previous struvite stone analyses (14). Pseudomonas, along with other microorganisms associated with urinary tract infections (e.g. *Proteus, Klebsiella and yeast*), produce ammonia and
increase urine alkalinity (pH > 7), which ultimately form struvite infection stones (14, 21). In addition, the present study identified fungal sequences affiliated with *Aspergillus* and *Candida* preserved within CaOx, brushite and struvite stone fragment groups (Figures 3b, d, and f). This is the first amplicon gene sequencing identification of fungi in these three mineralogies and is consistent with previous culturing studies that documented *Candida* growth from powdered CaOx stone fragments (9).

It has also recently been observed that idiopathic stone forming patients have developed an imbalance in the normal healthy composition of the bacterial communities inhabiting the gut (*dysbiosis*) (17, 19, 22). These dysbiosis studies have focused on gut microbiome networks that include the oxalate-degrading anaerobic bacterium *Oxalobacter formigenes*, whose activity is strongly controlled by changes in patient diet (19, 23). The metabolic activity of *Oxalobacter formigenes* causes oxalate degradation, which decreases oxalate concentration in the serum to make intratubular CaOx precipitation less likely (24, 25). Alternatively, enzymatic reactions might effectively degrade oxalate without the presence of *Oxalobacter formigenes*, which would similarly serve to decrease enteric hyperoxaluria (26-28). In addition to CaOx stones, hypercalciuria and elevated urine pH in brushite stones may also result from altered metabolic activity of the gut microbiome (12, 29).

Antibiotics, probiotics and microbial transplants further impact *Oxalobacter formigenes* colonization (17). Hyperoxaluria and CaOx urine supersaturation have also been shown to be attenuated by probiotic treatment (5, 12, 30). These studies have led to the hypothesis that the gut microbiome (27) acts as a metabolic modulator that directly
influences CaOx kidney stone formation, creating a connection within the human body called the gut-kidney axis (19, 31).

**Biomineralization in Human Kidneys and Natural and Man-Made Environments**

Microbial communities play a fundamental role in the precipitation, dissolution and recrystallization of phosphate, carbonate and silicate biomineralization in natural and man-made environments around the world (e.g. coral reefs, hot springs, deep subsurface; Figure 9) (2). In these settings, cell wall surfaces, extracellular polymeric substances and other organic molecules produced and influenced by microbial metabolism can act as direct surface control on the rate and composition of crystalline mineral growth (6). *In situ* kinetic experiments in Yellowstone National Park carbonate hot springs have demonstrated that the physical cell presence and biomolecules produced by living microorganisms can more than double mineral precipitation rates compared to when they are absent (32). These direct catalytic influences (*protein catalysis*) (33) can now be linked to microbial gene targets identified by future microscopy-to-omics analyses in kidney stones. Additionally, microbes also cause dissolution across a wide range of rock types, most commonly via secretion of metabolic byproducts such as organic acids and production of biofilms. These processes, which make significant contributions to biogeochemical cycles in everything from natural rock formation to building stone degradation, have previously been well documented in both field and controlled laboratory conditions (34). Overall, microbially-mediated mineral precipitation and dissolution is ubiquitous on Earth (6).

Initially, microbial populations are directly influenced by the environmental conditions in which they live (35). However, microbial populations can also rise to control the
surrounding physical, chemical, and biological environment (6). Similar types of dynamic feedback may occur during human kidney stone formation. This include interactions amongst kidney physiology, microbiome community structure, hydraulic properties, biogeochemical composition of the urine (e.g. chemistry, temperature, pH, saturation, flow rate, nutrient substrates) and the biomolecule composition and reactivity of internal renal surfaces (e.g. tissues, stones, biofilms) (3). Acting in concert, these key factors of the renal environment will impact numerous aspects of kidney stone formation, including stone crystalline architecture, mineralogy, rate of crystallization, and timing and extent dissolution. As a result, microbiome-driven biomineralization and dissolution processes within the human kidney likely play a significant role in kidney stone formation. These insights can now be utilized to generate new testable hypotheses for mechanisms of the microbially-mediated diagenetic phase transitions that may be intimately involved in kidney stone formation.

Microbial communities can play vitally important roles in the bulk concentration of ions (e.g., changes in fluid urine chemistry and kidney stone mineralogy and crystallography) that may in turn modify nucleation, crystallization, aggregation and dissolution during the overall history of stone growth (2, 3). An example of this is struvite stone formation, which has already been shown to be due to the effects of urine alkalinity generated by the metabolic activity of urease-producing bacteria (14). The similarities between kidney stones and rocks in the natural environment, as revealed in the present study and previous studies, can now be used to develop new hypotheses regarding their formation. For example, the partitioning of fungal and bacterial communities between CaOx, brushite, and struvite stone types is analogous to that found in many other natural rock formations such as hot spring travertine (6) and marine coral skeletons.
(Figure 9) (36). As another example, kidney stones contain entombed organic matrices (proteins, lipids and glycosaminoglycans) that are derived from the host human kidney, the microbiome, and urine (37-39). Organic matrices entombed within mineral deposits are observed in many other common examples of biomineralization such as in human bones, coral reefs, and pearls (40-43). These biomolecules will play a similarly crucial role in both promotion and inhibition of crystal growth and dissolution during kidney stone formation (4, 44). In the natural environment, fungal species such as *A. niger* ubiquitously mediate diagenetic phase transitions (45, 46).

**Conclusions**

These microscopy-to-omics results pave the way for future experimentation, in which these hypotheses can be systematically tested using highly-controlled microfluidic devices (4, 47). For instance, microfluidic testbeds can be used to quantitatively track the intermediate steps of kidney stone formation in real time under high-resolution microscopy, as in a recently published reports (2, 3, 47). This would allow specific mechanisms and processes controlling kidney stone growth and dissolution to be tested in the context of microbiome community, phylogenetic diversity, functional activity, and biochemistry. Examples include determination of the extent to which Tamm-Horsfall and other proteins serve to promote or inhibit stone growth, while also evaluating its role in protecting the urinary tract and other human organs from fungal infections (4, 11, 20, 25). Other experiments might include tracking stone growth rate, crystalline structure and mineralogy while changing the microbiome to reflect actual urinary microbiome community diversity, structure and metabolism (11, 20, 25). This could include mimicking the dysbiosis observed in urine from the renal calyx and bladder of calcium-based male stone formers (11, 20, 25). The effect of all types of microbial imbalances
on kidney stone growth could therefore be tested, including those resulting from changes in the gut and urinary microbiome due to diet, lifestyle, and frequent antibiotic use (22, 48).

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All authors read and approved the final manuscript.

Supplementary Material Contents

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**Figures and Captions**

**Figure 1. Study Flow.** A total of 20 patients were consented and enrolled in the study. Mineralogies analyzed in the present include CaOx ($n = 18$ patients), struvite ($n = 1$ patient) and brushite ($n = 1$ patient). After filtering reads through pipelines for quality, preprocessing and contamination, *16S* rRNA sequences (V3-V5 hypervariable region) were detected in 9/20 patients (45%), while †ITS2 sequences were detected in 11/20 patients (55%).

**Figure 2. Total number of reads and taxa grouped by kidney stone mineralogical type.** Number of amplicon sequence variants (ASVs) detected within calcium oxalate (CaOx), brushite and struvite kidney stones, respectively, targeting the (a) V3-V5 hypervariable region of the 16S rDNA gene and (b) ITS2 hypervariable region of the fungal rDNA gene. The number of reads for each individual patient stone fragment group are presented in Supplementary Figure S1. The symbol "*" represents 6 of the stone fragment groups presented in Figure 2a, and an additional 3 stone fragment groups included in calculation. † Represents the same stone fragment groups presented in Figure 2a. (c) Binary heatmap indicating the presence or absence of unique taxonomical divisions identified from ASVs (blue = bacteria, gray = non-ribosomal host human DNA, green = fungi). n-values correspond to individual patient stone fragment groups.

**Figure 3. Phylogenetic diversity of three mineralogical types of human kidney stones.** Pie charts represent the community diversity of bacterial 16S rRNA gene sequences, non-ribosomal human host (a, c, e), and ITS fungal sequences (b, d, f)
from CaOx, brushite and struvite patient-specific kidney stone fragment groups, respectively. The community diversity of bacterial and fungal sequences for each individual patient kidney stone fragment group is presented in Supplementary Figure S1.

**Figure 4. Patient metadata analysis.** Wilcoxon signed-rank tests indicate that the detection of fungal sequences is not significantly correlated with elevated urine calcium excretion at the $\alpha = 0.05$ level ($p = 0.07$). No statistically significant correlation was observed between the presence or absence of fungal sequences and urine oxalate excretion. Mean and standard deviation for each sample group is depicted in the text below the graphs. Purple and blue boxes are box plots for urine calcium and oxalate levels, respectively. The box plot summary statistics are as follows: lower boundary of box = first quartile, line in center of box = second quartile (median), upper boundary of box = third quartile. Dots represents individual patient data points. A dot unattached to the vertical lines represents outlier points, which were included when calculating the interquartile range.

**Figure 5. Microscopy evidence of microorganism entombment within human kidney stones.** Mineralogical identifications determined with a combination of bulk stone Fourier Transform Infrared (FTIR) analyses and determination of individual crystal morphologies and Raman spectroscopy (Figure 7) in thin section. (a), TPMT (b), and SRAF (c) TPMT overlaid on SRAF image from a struvite kidney stone documenting entombed bright orange auto-fluorescent coccoidal and rod-shaped bacterial cells. See also Figure S5 for contextualization of the occurrence of both coccoidal and rod-shaped bacteria in surrounding regions of the thin section.
Figure 6. Evidence for bacteria entombed within struvite and brushite kidney stones. Mineralogical identifications determined with a combination of bulk stone Fourier Transform Infrared (FTIR) analyses and determination of individual crystal morphologies\(^2\) and Raman spectroscopy (Figure 7) in thin section. (a) Color brightfield (BF) image of a 25 µm-thick thin section prepared from a struvite stone. (b) The same field of view as in a indicating that concentrically-layered spherulitic hydroxyapatite exhibits extinction under polarized light (POL) and are therefore amorphous (non-crystalline). Conversely, the radiating needle-like (acicular) crystals of struvite are strongly birefringent. (c) Color brightfield (BF) image of enlargement box shown in a. White arrows in c and d indicate cross sections at various oblique angles of entombed coccoidal and rod-shaped bacteria. (d) Superresolution autofluorescence (SRAF) image of the same field of view shown in c. (e) BF image of polymorphic twinning of radiating acicular brushite crystals. This is a \(~600\) nm thick slice in reflection mode, therefore not all BF objects are not in focus. White arrows in e and f indicate cross sections at various oblique angles of entombed coccoidal and rod-shaped bacteria. (f) SRAF image of the same field of view shown in e. Regions of blurred (fuzzy) concentric zonations represent regions of mimetic dissolution and replacement\(^2,4\).

Figure 7. Raman spectroscopy evidence for entombed bacteria within a struvite (\(\text{NH}_4\text{MgPO}_4\cdot6\text{H}_2\text{O}\)) human kidney stone. (a-c) Mineral component 1 (a, pseudo-colored red), mineral component 2 (b, pseudo-colored blue), and the corresponding merged images extracted from Raman spectra (c). (d) Transparent overlay of image c on a lower magnification color brightfield (BF) image illustrating optical microscopy correlated with Raman spectroscopy. (e) Raman spectra for mineral components 1 (a)
and 2 (b) with legends highlighting chemical components for identified peaks based on Takasaki(S11) and Balan et al(S12). (f-j) Enlargement of box in (d) similar to figure 5c but with high magnification Raman scan psuedocolored red for hydroxyapatite Ca$_{10}$(PO$_4$)$_6$(OH$_2$) and green for struvite. Note the 959 peak (italicized in j) is the ‘high’ peak for hydroxyapatite among the other peaks. Also note the similarity between struvite peaks in e (larger field of view) and j (smaller field of view).

**Figure 8. Microscopy evidence for entombed bacteria in brushite human kidney stones.** (a) Color brightfield (BF) image of a 25 µm-thick thin section prepared from a brushite stone. (b) Same field of view as in a indicating that concentrically-layered spherules exhibit extinction under polarized light (POL) and are therefore amorphous (non-crystalline; white arrows). Conversely, the radiating acicular crystals of brushite (white box in b, see also Fig. 5 e and f) are strongly birefringent. Inset in lower right in b is a TPMT image of spherules with entombed coccoidal and rod-shaped bacteria throughout each crystal (white arrows). White arrows in b indicate cross sections at various oblique angles of entombed coccoidal and rod-shaped bacteria.

**Figure 9. Microscopy-to-Omics evidence for microbiome entombment during biomineralization in natural environments.** Images and figures modified from previous publications$^{7,32,59}$. (a) A skeleton of the scleractinian coral *Orbicella annularis* from the leeward reef tract of Curaçao exhibits extensive fungal hyphae and borings (white arrow) in brightfield (BF). (b) Phase contrast (PC) image of the same field of view shown in a. (c) Rapid growth and accretion of CaCO$_3$ travertine at Mammoth Hot Springs in Yellowstone National Park is coated by and entombs coccoidal, rod and filaments of the bacterium *Sulfurihydrogenibium yellowstonense*. A merged blue and red
superresolution autofluorescence (SRAF) image (violet to red color) overlaid on a BF image. (d) Environmental scanning electron microscope (ESEM) of the same sample shown in c. (e) Phylogenetic diversity pie chart of the microbiome associated the deposition of hot spring travertine at Mammoth Hot Springs. (f) Phylogenetic diversity pie chart of the microbiome entombed in all calcium oxalate (CaOx) kidney stones analyzed in the present study.
Figure 1

Consent and enroll patients
\( n = 20 \) patients

Collect stone fragments from PCNL
\( n = 20 \) patients

Identify bulk mineralogy by IR spectroscopy
\( n = 20 \) patients

CaOx Patient Stone Fragment Groups
\( n \approx 18 \) patients

DNA extracted using PowerSoil DNA Isolation Kit
\( n = 18 \) patients

Petrographic thin sections available for imaging
\( n = 5 \) patients

16S rRNA gene sequences (V3-V5 hypervariable region) amplified on Fluidigm system and paired-end sequenced on an Illumina MiSeq platform
\( n = 9 \) patients

16S rRNA gene sequences (V3-V5 hypervariable region) passed quality control, pre-processing and contamination pipelines
\( n = 9 \) patients

16S rRNA gene sequences (V3-V5 hypervariable region) passed quality control, pre-processing and contamination pipelines
\( n = 7 \) patients

Internal transcribed spacer (ITS2) sequences amplified on Fluidigm system and paired-end sequenced on an Illumina MiSeq platform
\( n = 11 \) patients

Internal transcribed spacer (ITS2) sequences passed quality control, pre-processing and contamination pipelines
\( n = 1 \) patient

Brushite Patient Stone Fragment Group
\( n = 1 \) patient

DNA extracted using PowerSoil DNA Isolation Kit
\( n = 1 \) patient

Petrographic thin sections available for imaging
\( n = 1 \) patient

Struvite Patient Stone Fragment Group
\( n = 1 \) patient

DNA extracted using PowerSoil DNA Isolation Kit
\( n = 1 \) patient

Petrographic thin sections available for imaging
\( n = 1 \) patient

16S rRNA gene sequences (V3-V5 hypervariable region) amplified on Fluidigm system and paired-end sequenced on an Illumina MiSeq platform
\( n = 1 \) patient

16S rRNA gene sequences (V3-V5 hypervariable region) passed quality control, pre-processing and contamination pipelines
\( n = 1 \) patient

Internal transcribed spacer (ITS2) sequences amplified on Fluidigm system and paired-end sequenced on an Illumina MiSeq platform
\( n = 1 \) patient

Internal transcribed spacer (ITS2) sequences passed quality control, pre-processing and contamination pipelines
\( n = 1 \) patient

Internal transcribed spacer (ITS2) sequences passed quality control, pre-processing and contamination pipelines
\( n = 1 \) patient

Internal transcribed spacer (ITS2) sequences passed quality control, pre-processing and contamination pipelines
\( n = 1 \) patient

Internal transcribed spacer (ITS2) sequences passed quality control, pre-processing and contamination pipelines
\( n = 1 \) patient
Figure 4

**Urine Calcium (mg/24 hours)**
- **Absent**
  - Sample Size: 7 patients
  - Mean: 183.0 mg/24 hrs
  - Standard Deviation: 114.3 mg/24 hrs
- **Present**
  - Sample Size: 8 patients
  - Mean: 308.8 mg/24 hrs
  - Standard Deviation: 143.4 mg/24 hrs

**Urine Oxalate (mg/24 hours)**
- **Absent**
  - Sample Size: 7 patients
  - Mean: 33.0 mg/24 hrs
  - Standard Deviation: 22.8 mg/24 hrs
- **Present**
  - Sample Size: 8 patients
  - Mean: 20.2 mg/24 hrs
  - Standard Deviation: 12.8 mg/24 hrs
