



Calcium carbonate precipitation induced by calcifying bacteria in culture experiments: Influence of the medium on morphology and mineralogy

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ABSTRACT

Bacterially induced calcium carbonate precipitation, recognized as a common phenomenon in nature, has been extensively investigated owing to its beneficial and harmful effects on calcareous stones, namely environmental friendly and potential approach for consolidation deteriorated artworks and aesthetic and physicochemical damage to intact stones. Here four bacterial strains, identified as *Pseudomonas* sp. (N9), *Bacillus cereus* (T6), *Lysinibacillus sphaericus* (T5) and *Bacillus* sp. (T4), were isolated from white plaques covering historical white marble and proven to have the ability to induce precipitations of calcium carbonate. However, the size and morphology of crystals exhibit a distinct difference after inoculation of specific strain, even in the presence of same medium the crystals tend to vary with different isolates. XRD analysis shows that both calcite and vaterite were simultaneously present in strains N9- and T6-inoculated M-3P medium, while only calcite was detected in M-3P medium after inoculation with strains T5 and T4. Interestingly, in strains T6-and T5-inoculated B-4 culture medium, the precipitations were mainly composed of calcite and vaterite, with a small amount of weddellite only obtained from the former, while calcium-containing crystals were also absent from strains N9- and T4-inoculated medium. Moreover, all of the precipitated crystals present in both culture medium correspond to pH analyses.

1. Introduction

Increasing atmospheric pollution and biotic invasions have been endangering the survival of architectural and sculptural stones, exhibiting an excessive discoloration, weathering, mineralogy and structure damage (Rodriguez-Navarro and Sebastian, 1996; Urzi, 2004; Wakefield and Jones, 1998). Especially carbonate stones, are vulnerable to undergo abiotic and biological deterioration due to the composition and textural characteristics (Mitchell and Gu, 2000; Warscheid and Braams, 2000). Encrustation on the surface of calcareous stone is also a common phenomenon, which probably result from atmospheric particles being trapped in gypsum and making possible the crystallization of calcite (Saiz-Jimenez, 1997). Interestingly, another type of crust formation and incrustations on stonework, also known as microbially induced carbonate precipitation, is known to be a natural phenomenon associated with all kinds of microorganisms and commonly present in exposed calcium-containing stones (Li et al., 2015). Bacterially induced precipitation of CaCO₃, the so-called “carbonatogenesis”,

has aroused extensive attention owing to its significant potential application in conservation and consolidation of stones (Michaelsen et al., 2009; Rodriguez-Navarro et al., 2003).

Bacterially induced CaCO₃ precipitation is regarded as a complex process which involving environmental conditions and biotic metabolism pathway, both of which can influence the initial precipitation process, as well as overall accumulation of these deposits (Li et al., 2015). Bacteria can regulate precipitation by altering environmental parameters, on the one hand, creation of an alkaline environment via metabolic activities and increase of the concentration of dissolved inorganic carbon, on the other hand, control both the morphology and the mineralogy of the CaCO₃ crystals through producing extracellular polymeric substances (EPS) (Cassarino et al., 2003; Ercole et al., 2007; Giuffrè et al., 2013). Generally, the precipitation process can be simply described as: positively charged Ca²⁺ ions move into the cell surface and adsorption on anion of EPS, changes in cell surface charge allow interaction between other bacteria with different surface charge. Meanwhile, bacterially physiological activities and respiration

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functions lead to an increase in pH values and dissolved inorganic carbon, make a conversion of substantially soluble calcium ions to insoluble CaCO_3 precipitations (Beveridge and Fyfe, 1985; Beveridge and Murray, 1980). Despite a large number of studies on the bacterially induced CaCO_3 precipitation, the reason for the cause of polymorph selection is not clearly understood (Rodríguez-Navarro et al., 2012). It has been reported that specific EPS composition and structure, dissolved organic carbon release, bacterial types and culture medium also influence CaCO_3 crystals formation and control directly or indirectly calcite, aragonite and vaterite selection (Cassarino et al., 2003; Chen et al., 2009; Tournay and Ngwenya, 2009). Vaterite, is generally known as a most thermodynamically unstable polymorph and can easily change into calcite or aragonite in nature, but precipitated in the presence of biotic experiments (Bundelewa et al., 2012). As for the formation mechanisms of vaterite, many researchers believe that cellular EPS also play a decisive role in the processes of biomineralization (Cassarino et al., 2003; Ercole et al., 2007).

The objective of the study is to isolate and characterize calcifying bacteria able to induce calcium carbonate precipitation from calcareous stone monuments. Additionally, it is intended to evaluate the effects of the application of two calcium-containing medium on the morphological structure and composition of precipitated crystals. Our results will help to understand the formation of white plaque and make it possible to design an effective and environmental friendly method for *in situ* monumental stones consolidation.

2. Materials and methods

2.1. White plaques sampling and identification

The white plaques, showing morphologically irregular sediments or precipitations, were present in a column pedestal made of white marble (calcite), a remnant of imperial gardens in the Qing dynasty (1644–1911) and located in the West Lake Cultural Landscape site of Hangzhou, China. The samples were collected aseptically with sterilized scalpel, put in sterile Eppendorf tubes, then transported under refrigeration to laboratory and stored at 4 °C until analysed. The samples were divided into two groups: set 1 for bacterial isolation and set 2 for component analysis. To further analyze the plaques, several different methods were used in the analysis of chemical composition and crystal structure, namely SEM-EDS, μ -FTIR and XRD. The microtextural character of the precipitations were observed by Scanning Electron Microscopy (SEM) (Hitachi SU8010) equipped with Energy dispersive X ray spectrum (EDS). For the SEM analysis, samples were directly glued on double sided carbon adhesive tape and then coating was done with gold for 90 s. Structural characterization of samples were obtained by Nicolet iN5 FTIR spectrometers and microscopes (Thermo). X ray diffraction spectra (XRD) were obtained via Cu $K\alpha$ radiation (tube operating at 40 kV and 250 mA) with scintillation detector (Rigaku D/Max-2550pc) for identifying different crystalline phases of calcium contained precipitation formed. Survey data was analysed and comparing them with the standards (International Centre for Diffraction Data) to determine the chemical composition of samples.

2.2. Isolation and identification of calcifying bacteria

Dilution plate method disclosed for treating a mixed sample was utilized to isolate bacteria colonization on white plaques. In summary, around 50 mg of calcareous sample was powdered in sterile set of pestle mortar in a low temperature environment, then suspended in phosphate buffered saline (PBS) buffer (0.01 M, pH 7.4), briefly vortexed and submitted to serial dilutions ranging from 10^{-1} to 10^{-4} . Aliquots 100 μ l suspension of microbes and precipitation were spread on LB and Trypto-soy agar medium (TSA) agar medium plate supplemented with antifungal antibiotic nystatin and then incubated at 30 °C for several days until visible plaques appeared. Finally, the single strain from both

medium was selected based upon morphological differences and labelled individually. Molecular characterization of the bacterial isolates was done by harvesting the strains grown in LB for one day. Subsequent bacterial genomic DNA extraction was performed using a rapid CTAB DNA extraction following the previously reported protocol (Jr and Via, 1993). Afterwards, extracted genomic DNA was used as a template to amplify bacterial 16S rDNA gene fragments with the specified primer 341F and 907R. The amplification was performed in total volume of 50 μ l, containing 2 μ l DNA, 2.5 μ l of 10 μ M forward and reverse primer, 5 μ l PCR buffer, 4 μ l dNTPs, 0.5 μ l Taq polymerase, add water to a final volume of 50 μ l, with PCR reaction program: 94 °C for 4 min, followed by 30 cycles at 94 °C for 30 s, 53 °C for 30 s and 72 °C for 45 s, and the final extension at 72 °C for 10 min. The PCR products were further purified using QIAquick PCR Purification Kit and then sequenced directly by Sanger's dideoxy chain-termination sequencing method (Genewiz, Suzhou). The obtained 16S rDNA sequences were deposited in GenBank with accession numbers MH458751–458760. The sequences were then compared with the most closely related 16S rRNA gene sequences in the GenBank database and aligned using Clustal W (Thompson et al., 1994) and neighbor-joining phylogenetic trees were constructed in MEGA 7 (Kumar et al., 2016) with distances estimated by the Kimura 2-parameters model and a bootstrap of 1000 replicates.

For inoculum preparation, ten colonies were precultured in liquid LB medium and incubated on a shaker for 36 h at 30 °C, adjust the bacterial suspensions to an OD600 of between 0.5 and 1.0 with PBS buffer. Two mineralization medium are involved, including modified B-4 medium (2% calcium acetate, 1% yeast extract and 1% glucose and 2% agar, adjust pH to 8.0) (Boquet et al., 1973) and M-3P medium (1% Bacto Casitone, 1% $\text{Ca}(\text{CH}_3\text{COO})_2 \cdot 4\text{H}_2\text{O}$, 0.2% $\text{K}_2\text{CO}_3 \cdot 1/2\text{H}_2\text{O}$ in a 10 mM phosphate buffer, 2% agar, pH 8.0) (Rodríguez-Navarro et al., 2003). One hundred microliter of diluted bacterial suspensions of 10 isolates incubated at 30 °C in B-4 and M-3P solid medium, while the control group consisted of sterilized strains inoculated with medium under the same culture conditions. A minimum of two petri dishes were run in each assay and these plates were incubated at 30 °C under static culture for 20 days. The mixture of precipitated carbonates and bacterial cells were wiped with aseptic swab and spread on slides for microscopic observation. Calcein, known as a fluorescent marker that binds to calcium and is readily incorporated into newly precipitated calcium-containing crystals (Moran, 2000; Tambutté et al., 2012), was used to determine the presence of calcium in precipitated crystals with a fluorescent microscope (blue light excitation). Selected isolates, tending to have strong calcification activity, were further used to carry out bacterially induced carbonate mineralization experiment.

2.3. SEM and XRD analysis of precipitated CaCO_3 crystals

Calcium-containing crystals precipitated by selected isolates N9, T6, T5 and T4 were collected after 20 days of incubation in M-3P and B-4 solid and liquid medium and placed into sterilized tubes. For analyses of morphological structure of crystals, cells deposits were washed twice with sterile deionized water and centrifuged for 10 min at 6000 rpm. After the supernatant was removed, the collected crystals was then distributed equally on slides and observed by optical microscopy. Scanning electron microscopy was carried out with crystals precipitated in M-3P and B-4 liquid medium of four isolates at 30 °C after 20 days in culture. Washed carbonate crystals and bacterial suspension were spread on conductive adhesive and gold coated prior to observation. The morphological structure of precipitated crystals in contact with the bacterial strains and extracellular polymeric substances (EPS) were observed. For XRD qualitative analysis of the newly formed carbonates, four different isolates samples in M-3P and B-4 liquid medium after 20 days in culture were prepared. XRD measurements were made on a scintillation detector (Rigaku D/Max-2550pc). Samples were placed in the XRD sample holder without any prior grinding or preparation.

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