Bacterial mineralization of calcium carbonate for conservation of stone artworks

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Abstract. Calcareous stones have been widely used as artwork and building materials by human civilizations, especially in the Mediterranean Basin. Calcareous stone deterioration is a consequence of the weathering action of physical, chemical and biological factors, which causes a progressive dissolution of the mineral matrix and an increase in porosity, resulting in the weakening of the structure. Recently, increased environmental pollution and global warming are threatening stone cultural heritage more than ever. Inorganic or organic products have been using to slow down monument deterioration, but their use presents several drawbacks. Bacterial Calcium Carbonate Mineralization is a natural process widespread among bacteria and occurring in different environments. In the last decades it has been proposed as a new and environmentally friendly tool in conservation of monumental and ornamental calcareous stones. The aim is to develop a bacteria-mediated mineralization treatment providing a coherent calcium carbonate layer on the surface of deteriorated stone, able to protect it against the intake of water and chemicals and to consolidate the inner weakening structure. The advantage is to obtain a mineral product similar to the calcareous stone substrate, mimicking the natural process responsible for stone formation. This review introduces the mechanisms of bacterial mineralization and describes the current strategies based on this process to promote stone reinforcement in field tests. They include applications of selected bacterial strains and/or culture media as well as selected components of bacterial cells on stone. The review finally provides perspectives based on recent advances.

1. Bacterial Calcium Carbonate Mineralization
Bacterial Calcium Carbonate Mineralization (BCCM) is a major biogeochemical process widespread among bacteria in different environments such as marine waters and sediments, freshwater, and soils [1]. According to [2], “under suitable conditions most bacteria form calcite crystals”. Different bacterial species are capable of precipitating different amounts, shapes and types of carbonate crystals [3]. BCCM represents a fundamental part of the calcium as well as of the carbon biogeochemical cycle, contributing to carbon sequestration and formation of calcium carbonate (CC) sediments, deposits and rocks [4].

Different mechanisms of bacterial involvement in calcification have been proposed [5]. The studies made in this field have pointed out the complexity of the phenomenon that can be influenced by the environmental physico-chemical conditions and it is correlated both to the metabolic activity and the cell surface structures of bacteria. According to [3], as a chemical process, BCCM has governed by four key factors: (1) the calcium (Ca\(^{2+}\)) concentration, (2) the concentration of dissolved inorganic carbon (DIC), (3) the pH and (4) the availability of nucleation sites. Bacteria can change the surrounding environment by increasing DIC and shifting the pH towards alkalinity through different physiological activities and, at the same time, offer nucleation sites for mineralization [5]. The occurrence of these conditions can, in presence of calcium ions, foster BCCM when a state of oversaturation develops [6].

Metabolic pathways involved in BCCP include autotrophic as well as heterotrophic pathways with a different contribution. The heterotrophic pathways belonging to the nitrogen cycle are those mainly used for applications of BCCM. They include ammonification of amino acids, dissimilatory reduction.
of nitrate, and degradation of urea or uric acid [7]. These pathways induce production of carbonate and bicarbonate ions and, as a metabolic end-product, ammonia, which causes a pH increase. When the H+ concentration decreases, the carbonate-bicarbonate equilibria are shifted towards the production of CO3²⁻ ions (reaction 1). If calcium ions are present, BCCM occurs (reaction 2).

$$\text{(1)} \quad \text{HCO}_3^- \leftrightarrow \text{CO}_3^{2-} + \text{H}^+$$
$$\text{(2)} \quad \text{Ca}^{2+} + \text{CO}_3^{2-} \leftrightarrow \text{CaCO}_3$$

Bacterial surfaces also play an important role in CC precipitation. Cell surface structures of bacterial cells, primarily cell walls, can act as important sites for the absorption of cations as Ca²⁺ and constitute particularly favourable templates for heterogeneous nucleation and crystal growth [6]. Surface macromolecules can play this role both as part of bacterial cell and as cell-free, when released in the environment; in the latter case primarily as extracellular polymeric substances or EPS. EPS is a broad term that groups a large variety of organic polymers, mainly polysaccharides, secreted by microbial cells [8]. The role of EPS in CC nucleation and growth is well documented in laboratory experiments on bacterial cultures or isolated EPS as well as in natural environments such as microbial mats [9]. Bacterial EPS have been shown to act as matrix template not only by entrapping ions and serving as a nucleation site but also by the action of specific proteins that influence CaCO₃ morphology, polymorphism, spatial position and growth (see references in [10]). The organic matrix is trapped in the growing CC crystals [11]. According to [8], a common location for EPS is the microbial “biofilm”. In fact, the organic EPS matrix is an integral part of the ubiquitous microbial biofilms. In the biofilm microenvironment, precipitation exhibits some spatial organization and the EPS may represent a primitive regulation of precipitation, for example in lithifying mats of marine stromatolites. The biofilm and its associated EPS matrix could therefore serve as a useful starting point to investigate how organic molecules influence the precipitation process.

Carbonate precipitates commonly start to build up and develop on the external surface of bacterial cells, accordingly to the bacterial cell acting as heterogeneous crystallization center, and bacteria get embedded into the growing carbonate minerals [7, 12]. The polymorph of CaCO₃ formed (calcite, aragonite or vaterite) depends both on the environmental conditions and the bacterial strain [5, 12]. The most common crystalline forms are either spherical or polyhedral.

BCCM has drawn much attention in recent decades because of its potentiality in numerous applications. Proposed innovative applications include biomimetic processes and materials, solid-phase capture of inorganic contaminants, cementation of soil and construction materials, and protection of monumental calcareous stones [13, 14, 15, 16].

2. BCCM-based strategies for conservation of stone artworks

Monumental stone decay is a consequence of the weathering action of several physical, chemical and biological factors, which induces a progressive dissolution of the mineral matrix. In the case of calcareous stones, the material, due to calcite leaching, increases in time its porosity and decreases its mechanical characteristics [17]. Application of conservative treatments with inorganic or organic products have been using to slow down the deterioration of monuments, but their use presents several drawbacks [13]. Organic products have a chemical composition and a thermal expansion coefficient which are quite different from that of the stone. Besides, they are usually applied in organic solvent which is dispersed in the environment. Furthermore, the efficiency in time of these treatments is short and, in some cases, they can have a detrimental effect for the conservation of the stone itself. On the other hand, inorganic consolidants show poor performance under field conditions [13].

During the last decades, BCCM has been proposed as a new environmentally friendly tool for the protection and reinforcement of monumental and ornamental calcareous stones [18]. The advantage of a BCCM-mediated treatment is to obtain a mineral product inside stone pores similar to the substrate,
mimicking the natural process responsible for stone formation. The aim is to provide a coherent CaCO$_3$ layer on the surface of deteriorated stone, protecting it against the intake of water and chemicals and consolidating the inner weakened structure. According to the current state-of-the-art, there are three strategies exploiting the BCCM technology to treat stone monuments in situ: application of living cultures of selected bacterial strains, application of bacterial culture media, and application of selected components of bacterial cells.

The application of BCCM for cultural heritage stone conservation was initially proposed by a French group who made a pioneer work and developed the so-called Calcite Bioconcept technology, covered by a patent now expired [18]. The technology was based on the application of cultures of selected bio-calcifying bacterial strains. They were sprayed on the stone surface and then fed with a suitable growth medium. As result, a new calcareous coating layer, called biocalcin, formed. It was few µm thick, coherent to stone and constituted of encrusted bacterial bodies mixed with CC. Preliminary work was done in laboratory conditions to test bacteria isolated from natural carbonate environments and suitable media. Then the process was simulated on miniature walls of two kinds of limestones at different porosity. Bacillus cereus, showing the highest precipitation performance among the screened bacteria, was selected for life-size applications. The treatment consisted of first spraying the stone surface with the bacterial suspension culture. Afterward, bacteria were fed for the next four days with a nutrient medium sprayed on stone [18]. Nutrients applied stimulated the metabolic pathway of ammonification of amino acids linked to the nitrogen cycle and driving precipitation. The first application in situ was tested in 1993 on an area of 50 m$^2$ of the tower of the Saint Médard Church in Thouars. The treatment was evaluated 6 months and 1 year after the application by measuring water absorption and color of stone surface. According to the authors, the water absorption rate decreased up to 5 times, accordingly to the biocalcin deposition inside stone pores; no changes in color or other aesthetic appearances were detected [18]. Following this approach, a number of façades of French historic and private buildings have been treated with this method by the Company Calcite Bioconcept [13]; nevertheless, no scientific reports are available about these treatments. After that, several groups have worked to improve this process mainly in laboratory conditions, obtaining to some extent similar results [19].

A Spanish group at the University of Granada improved this technology and carried out field trials. Initially, they selected Mixococcus xanthus as calcifying microorganism, a Gram-negative, non-pathogenic soil bacterium, and tested it in laboratory conditions. Sterilized calcarenite slabs were immersed in a liquid medium containing nutrients promoting ammonification of amino acids and inoculated with $M$. xanthus. A new coherent carbonate cement of calcite grains formed into the pores to a depth $\geq 500$ µm [20], constituting a thicker layer than biocalcin. A step further in the development of this technology was the activation of the autochthonous CaCO$_3$ producing-bacteria inhabiting the stone without introducing any exogenous microorganism. In preliminary bio mineralization experiments, quarry porous limestone slabs were immersed in a nutritive buffered solution, called M-3P, inoculated and non-inoculated with $M$. xanthus [20]. Treated stones showed similar results regardless of the presence or absence of $M$. xanthus: newly precipitated CC without pore plugging and, accordingly, weight increase. Moreover, treated slabs resulted more resistant to mechanical stress than the sterilized slabs used as control, as demonstrated by the weight loss measurement of stones after sonication [20]. The M-3P medium, containing a calcium source and activating the carbonatogenetic bacterial community via the ammonification of amino acids, was patented. The new bio-consolidation treatment was then tested in situ on selected areas of deteriorated calcarenite stone of three historic buildings in Granada [21]. At the San Jerónimo Monastery and the Royal Hospital, the M-3P was applied both inoculated and non-inoculated with $M$. xanthus on flat surfaces of calcarenite stone blocks, to compare the two treatments. At the Royal Chapel, the only M-3P treatment was applied on elements of the crest with carved surfaces. All treatments were applied by spray for 6 days. The $M$. xanthus treatment was performed by two initial applications with the
bacterial culture (∼10⁹ cells/ml), followed by successive applications with the only M-3P medium. [21]. The evaluation of treatments included both the technical efficacy and, for the first time, the response of the bacterial community present in the decayed stone. Biodeposition was evaluated by analysing the new calcite microtexture, the consolidation effect by measuring surface cohesion, and the stone microbiota was monitored with culture-dependent (viable title of bacteria) as well as culture-independent techniques (total DNA, 16S rDNA amplification, DGGE and clone libraries). Medium/long-term efficacy and detrimental side-effects (included color and the impact on stone microbiota) were monitored up to 4 years after treatment. In all the three trials, newly formed CC (mostly calcite) deposited as a cement on stone surface without plugging pores nor detracting from its aesthetic appearance. This bio-cement consolidated the deteriorated calcarenite with a significant surface strengthening. The treatment efficacy in situ was independent of the presence of M. xanthus [21], as already found in laboratory conditions. Microbiological analyses showed that bacteria activated by the application of M-3P medium were able to produce CC. The bacterial population initially increased after treatment application but tended to reach values close to those observed before treatment over time [21].

The same authors proposed a variation of this technology called “self-inoculation” biotreatment. It is based on the isolation and selection of carbonatogenic bacteria from calcareous stone to prepare a bacterial inoculum that may be used in those cases where the stone microbiota has been altered and/or suppressed (e.g., by the use of biocides). The treatment was firstly tested in laboratory conditions, then the bacterial community resident on salt damaged carbonate stone in San Jeronimo Monastery was isolated and activated via M-3P, and applied back onto the same stone in situ [22]. The inoculum was constituted by Firmicutes as dominant phylum (∼79%), Gamma-proteobacteria (∼16%), and Beta-proteobacteria (∼5%). The self-inoculation treatment was compared with the M. xanthus and the sterile M-3P treatments. All three treatments were applied by spraying on stone blocks with similar exposure and decay levels for 6 days. The bacterial cultures (M. xanthus and self-inoculation treatments) were applied on stone on the first day, then the only M-3P medium was applied [22]. Biodeposition was evaluated by analysis of new calcite and of porosity and pore size distribution; bioconsolidation by measuring surface cohesion and cohesion profiles; safety of treatment by measuring color and monitoring stone microbiota. According to the authors, an effective consolidation and protection was obtained due to the formation of an abundant and exceptionally strong hybrid cement consisting of nanostructured CC and bacterial EPS covering the substrate [22]. Evolution of culturable microbiota was monitored before and up to 24 months after the self-inoculation treatment: the viable titer increased after 5 months, then dropped back close to the pre-treatment value after 24 months.

The exploitation of the metabolic activity of living bacteria to drive CC mineralization requires the application of organic nutrients on stone in both cases of activation of allochthonous and autochthonous bacteria. This can promote the undesired growth of environmental microorganisms causing mineral changes due to metabolic by-products or appearance of stained patches on stone [23]. For these reasons, an Italian team of Florence tested a new BCCM-based approach in absence of viable cells [17]. The work aimed at identifying bacterial structures or molecules inducing precipitation by using the well-known Bacillus subtilis strain 168. The capability of bacterial dead cells to precipitate CC was tested in a solution assay with CaCl₂ as calcium source and in the presence of sublimation of ammonium carbonate as carbonate source and for alkalization of the closed environment. Dead cells were able to induce calcite formation (Figure 1A). Bacterial cells fractions, obtained with different fractionation methods, were then tested in the precipitation assay. A bacterial cell fraction containing the cell wall, called BCF, induced calcite formation (Figure 1B). No CC polymorphs different from calcite were found by X-ray diffraction.
**Figure 1. SEM micrographs of calcite crystals produced by** *B. subtilis* **dead cells and BCF.** A. Calcite crystals induced by dead cells; crystal growth seem to be promoted by bacterial bodies. B. Calcite crystal aggregates induced by BCF. (With permission from Perito et al., 2014).

*B. subtilis* dead cells, as well as BCF, were able to act as heterogeneous crystallization nuclei in liquid medium. The capacity of bacterial cell walls to uptake cations such as Ca$^{2+}$ and fostering heterogeneous nucleation is known and has been previously demonstrated for isolated *B. subtilis* walls [6]. According to [9], this process (precipitation occurring onto surface of inactive or dead cells as well as onto isolated cell components) can be referred to biologically influenced mineralization. BCF was stored as easy-to use lyophilized preparations which showed to maintain a long-lasting precipitation activity. The BCF solution was tested as cell-free treatment on stone specimens of Pietra d’Angera lithotype and then on selected areas of the main façade of the Angera Cathedral, a 6th century monumental site in Italy [17]. In the field test, lyophilized BCF was dissolved in a CaCl$_2$ solution, then added to a supersaturated calcium bicarbonate solution (Super C solution) for supplying calcium ions and CO$_2$. BCF in Super C and only Super C solution as control (REF) were applied by spray on stone surface of adjacent areas for three days. The second day of treatment the BCF and REF solutions were also supplemented with calcite nanoparticles to maintain supersaturation in the pore and increase calcium ions. The third day only SuperC plus nanoparticles solution was sprayed on both the areas [17]. Field evaluation was carried out before and four months after treatment. BCF treated area showed negligible color changes and, if compared with the REF control area, a significant decrease in water absorption (up to 6.8%) and a significant increase in hardness in the first 3 mm, as indicated by the cohesion profiles. These data fitted with the new CC biodeposition detected inside stone pores. These results indicated the potentiality for this application, even if, according to the authors, further experimentation is required to fully assess the treatment conditions for in situ applications.

3. **Conclusions and Perspectives**

Calcareous stones have been widely used since ancient time in artworks and buildings in the Mediterranean Basin and throughout the world. Increased environmental pollution and global warming are threatening stone cultural heritage more than ever. Due to its low impact on stone and environment, BCCM-based biotechnology presents an ecological alternative to chemical treatments. It produces a layer of CC compatible with the stone (grown on pre-existing CaCO$_3$ of similar origin), strongly attached to the surface and exerting consolidation.

Considering the literature of the last years, while many efforts have been doing on the CaCO$_3$ mineralization potential of different bacteria at laboratory scale, very few attempts have been made to test the technology in situ since the pioneer work of the French group in the nineties. Translating some of the promising results obtained in laboratory settings into real practical biorestoration applications was and still is the challenge for the immediate future [10, 24]. Nevertheless, a few companies have
already developed bioconsolidation products for cultural heritage based on the application of cultures of selected bacterial strains (Amonit, France; http://www.amonit.fr/fr/calcite_1) or of media stimulating stone microbiota (KBYO Biological, Spain; http://kbyobiological.com/en/).

In our opinion, all the different approaches described in this review are worth further development for field applications. A common technical weak point is in the reinforcement effect, not yet comparable to that of synthetic polymers. The performance of the treatment could be improved also selecting an appropriate stone lithotype. Another general comment concerns the heterogeneity of the tests used for treatment evaluation. Methods for short- and long-term evaluation of both consolidation effectiveness and safety of stone should be defined and standardized for comparing results and for metadata analyses. Evaluation should include the impact of treatment on stone microbiota.

As mentioned above, the activation of living bacteria, both allochthonous and autochthonous, requires the application of nutrients directly on stone to exploit microbial metabolism driving mineralization. The possibility of undesirable side-effects and risks to the stone needs to be carefully evaluated [10, 19]. Promotion of undesired microbial growth can produce formation of stained patches on stone [23], of EPS with pores plugging or mineral changes [24].

It is well known that microbes can strongly contribute to stone deterioration [25]. Changes in the stone microbial community structure and growth of unwanted microorganisms could be a risk factor, also on the long-term effects. It is very important to detect the microbial communities inhabiting stone before any treatments and monitor their dynamics after treatments by using methods which provide an in-depth analysis of the microbial community structure, fluctuations and metabolic potential. What we know about microbial communities inhabiting monumental stone mainly comes from studies where cultivation-dependent methods have been used and only in recent years Next Generation Sequencing and “omics” techniques have been applied to their investigation [10, 26, 27]. These latest studies suggest that the community structure of stone microbiota detected by “omics” is quite different from that cultivated in nutrient media fostering CC precipitation. Cultivation of bacteria from stone to test their CC mineralization potential by enrichment cultures techniques revealed mainly presence of strains belonging to Firmicutes, a phylum very poor represented in the same stone community detected by using high-throughput sequencing (see references in [10]).

Meta-omics technologies provide a wider view of the stone microbial communities structure allowing a more careful evaluation of the impact of treatment on the ecology of stone. Moreover, they allow the detection of the community biomineralization potential by identifying the presence of microorganisms known as CC-producing as well as the presence of metabolic pathways known as fostering CC mineralization [10]. At the same time, meta-omics techniques can also provide useful information to improve cultivation strategies to isolate potential carbonatogenic bacterial populations from calcareous environments.

On the other side, the advantage of the cell-free approach is to induce mineralization without the application of nutrients on stone, bypassing the drawbacks due to microbial growth. Moreover, interventions on the chemical environment governing precipitation to improve its performance are easier. Another advantage is that cell components are smaller than cells and would penetrate more in depth into stone pores. The preparation of the current BCF product is however more complex compared with those from alive cells strategies.

The development of the cell-free approach, as well as of the other BCCM-based treatments, depends on the improvement of our knowledge on bacterial mineralization. Very little is still known about the molecular mechanisms acting at the cell microscale of the BCCM process and their understanding is necessary for applied purposes [5]. B. subtilis was used with the aim to identify cellular fractions as well as genes and molecules playing a role in inducing precipitation [28], as found for mollusks [29]. Analysis of B. subtilis mutants impaired in CC mineralization suggested possible links with redox reactions of fatty acid metabolism, phospholipids membrane composition and surface properties [28, 30, 31, 32]. A better understanding of these metabolic switches could help in the identification of bacterial molecules involved in mineralization and, at the same time, in the enhancement of mineralization performance by bacteria.
Other targets for future work are the EPS or other bacterial molecules acting as a matrix able to promote mineralization. As described above, the high consolidation effectiveness found with the self-inoculation biotreatment was ascribed to the hybrid cement formed by incorporation of microorganisms and EPS within the nanostructured CaCO$_3$ [22]. Recent findings in the study of biofilm structure have shown that bacterial biofilms contain a robust internal mineral layer, composed of calcite, and a potential use of bacteria in designing rigid construction materials and altering crystal morphology and function has been proposed [33]. Research on biopolymer-mediated precipitation is still in its infancy [8] and further studies are needed to test different EPS or bacteria-based biomimetic molecules working as a matrix promoting calcite growth on stone. This would allow to reduce the complexity of organic matter to apply and increase its penetration inside stone pores, representing a step forward in the development of the BCCM-mediated technology.

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