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Carbonate Crystals Precipitated by Freshwater Bacteria and Their Use as a Limestone Consolidant[▽]

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Bacterial carbonate precipitation is known to be a natural phenomenon associated with a wide range of bacterial species. Recently, the ability of bacteria to produce carbonates has been studied for its value in the conservation of limestone monuments and concrete. This paper describes investigations of carbonate crystals precipitated by freshwater bacteria by means of histological (Loeffler's methylene blue and alcian blue–periodic acid-Schiff stain) and fluorescence (CTC [5-cyano-2,3-ditolyl tetrazolium chloride]) stains, determination of cell viability inside carbonate crystals, and pore size reduction in limestone by image analysis. Carbonate crystals were found to be composed of bacteria embedded in a matrix of neutral and acid polysaccharides. Cell viability inside the carbonate crystals decreased with time. On stone, bacteria were found to form carbonate crystals, with only a few bacteria remaining as isolated cells or as cell aggregates. Pore size was reduced by about 50%, but no blockage was detected. Taken together, the results of this research provide some reassurance to conservators that biocalcification by bacteria could be a safe consolidation tool in a restoration strategy for building stone conservation.

Carbonates in the form of the different calcium polymorphs and dolomite represent an important carbon reservoir, accounting for 78.5% of the total carbon concentration on earth (22, 23). Carbonate precipitation by bacteria, referred to as biocalcification, has been widely studied in the saline aquatic environment by Lipman (34), Morita (35), and Novitsky (37); in fresh and saline soil by Parraga et al. (39), Ferrer et al. (24, 25), Rivadeneyra et al. (48, 52, 53, 54, 55), and Braissant et al. (10); and in freshwater environments by Danielli and Edington (17), Cacchio et al. (12, 13), and Baskar et al. (5). The ability of bacteria to precipitate carbonate crystals has been described by Boquet et al. (9) as a process that is widely spread among different bacterial species.

Most bacterial species are able to precipitate carbonates given the right conditions, namely, an alkaline environment rich in Ca^{2+} ions. Carbonate precipitation by bacteria is a complex process which was described by Castanier et al. (15) as involving metabolic pathways associated with photosynthesis, nitrogen and sulfur cycles, and ion exchange (Ca^{2+}/H^{+}) (29). Barabesi et al. (4) and Perito et al. (41) identified six genes involved in crystal formation in *Bacillus subtilis* PB19. The function of four genes (*yusJ*, *yusK*, *yusL*, and *ysiB*) was seen to be involved in fatty acid metabolism. Two others were found to be involved in membrane transport protein (*ykoY*) and regulation of transcription (TetR/AcrR family) (*ysiA*).

A step in the precipitation of calcium carbonate by bacteria has been described as involving stoichiometric interactions between positively charged Ca^{2+} ions and the negatively charged bacterial cell wall (7, 8). These Ca^{2+} ion-cell wall interactions produce changes in the overall charge of the cell wall, allowing interaction between differently charged bacteria. As a consequence of these changes in the overall ionic charge, bacteria aggregate to increase the size of the biomineral, and in turn, bacteria become the nucleus of the biomineral (25, 49, 51). Extracellular polymeric substances (EPS) produced by bacteria have also been shown to be involved in the process of biocalcification by entrapping and serving as a nucleation site (26, 60). In carbonate formation by *Pseudomonas fluorescens*, precipitation was seen to occur through the activity of a peptide matrix, with aspartic acid, glutamic acid, alanine, and glycine as the major contributing amino acids (2). Changes in the composition and concentration of EPS have an influence on both the morphology and the mineralogy of the carbonate crystals (10). EPS also appear to be involved in increasing the size of the crystals by causing the aggregation of smaller crystals (11).

The ability of *B. pasteurii* to precipitate carbonates has been previously investigated with respect to consolidation of sand columns (27, 59) and repair of concrete cracks (3). Recently, De Muynck et al. (19, 20) studied the possibilities of biocalcification by *B. sphaericus* as a concrete bioconsolidant. In the field of stone conservation, the ability of different bacterial species, such as *B. pasteurii* (33), *Myxococcus xanthus* (56), and *B. sphaericus* and *B. lentus* (21), to act as limestone consolidants has also been investigated.

The survival of bacteria inside the carbonate crystal could have serious implications for the use of bacterial calcification as a method of consolidation in conservation, because it has already been demonstrated that uncontrolled bacterial growth can damage stone (38, 42, 57, 61, 64). However, the controlled action of bacteria has been demonstrated to be potentially useful in the restoration of art works (14, 33, 44, 45, 46, 47).

Although there is agreement that bacteria become the nucleus of the precipitated carbonate crystal, as clearly shown by Rivadeneyra et al. (49 [see images]), there is still some uncer-

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tainty about bacterial survival inside the carbonate crystal. Greenfield (28) found one or two dead bacteria inside the carbonate crystals after dissolution of the crystals with HCl. Later, Krumbein (32), studying aragonite crystals, showed that "more than 20" living bacteria could be found in carbonate crystals 90 h following resuscitation but reported that they were dead after 7 days. Morita (35) found that a large number of bacteria were detectable inside carbonate crystals, although it was not specified whether they were viable or not. However, Parsek and Singh (40) found that bacteria inside kidney stones were viable and that while they were inside the crystals they were unaffected by antibiotic treatment. Most studies have been concerned with bacteria from marine or saline environments, and the circumstances of bacterial survival as well as the methods of investigation have not been clear.

This paper is concerned with investigation of microbiological techniques for a safe and effective application in stone preservation programs. The work presented aims to establish (i) what happens to bacteria during biocalcification and (ii) the impact that the crystals have on stone when used as a stone consolidant. The research therefore addresses three questions. (i) Where are the bacteria found in the newly precipitated bioliths? (ii) How long do bacteria remain viable inside them? (iii) Can we quantify the impact of biocalcification on limestone?

Thus, the evidence presented is intended to demonstrate the structure of crystals produced by biomineralisation, the extent to which bacteria survive, and the impact of the bacterial presence on the porosity of limestone. The overall aim is to inform the risk evaluation process for biotechnological treatment of heritage stone.

MATERIALS AND METHODS

Bacterial isolates. The bacterial isolates used in this study belong to the genera *Pseudomonas* (isolates D2 and F2) and *Acinetobacter* (isolate B14). All bacterial species were isolated from a calcified branch submerged in a freshwater stream in Somerset, England. These bacterial isolates have been previously described by Zamarreño et al. (63).

Production and collection of carbonate crystals. Bacteria were grown aerobically in 50 ml of modified B4 liquid medium (9) containing 5 g of calcium acetate, 1 g of yeast extract, and 1 g of glucose per liter in 100-ml conical flasks. The medium was adjusted to pH 8 with 1 M sodium hydroxide (NaOH). All bacterial strains were incubated at 30°C for up to 3 weeks. The experiments were carried out in triplicate; controls consisted of uninoculated B4 liquid medium and heat-killed bacteria. All chemicals were obtained from Fisher Scientific, United Kingdom.

The precipitated carbonates were collected on Whatman No. 1 filter paper (Fisher Scientific, United Kingdom) (90 mm in diameter) by filtration, washed with sterile distilled water (dH_2O), and air dried at 37°C for 48 h. The filters were weighed before and after collection of crystals to estimate the amounts of carbonate crystals precipitated by the different isolates.

XRD. X-ray diffraction (XRD) was used to identify the types of carbonate polymorph that were precipitated. XRD analysis was carried out with crystals precipitated in cultures of isolates B14, D2, and F2 after the third week of incubation. The qualitative analysis of the precipitated carbonate crystals was carried out by XRD with a monochromator Philips PW 1050/25 goniometer (Cu X-ray tube; $K\alpha$ radiation = 1.542 Å; 40 kV; 20 mA with a continuous scan of 2θ) attached to a Philips PW 1729 X-ray generator.

SEM. Scanning electron microscopy (SEM) was carried out with crystals precipitated in cultures of isolates B14, D2, and F2 at 30°C during the first week of incubation. The collected carbonate crystals were sputter-coated with a gold-palladium mixture (SEM E5000 coating unit). The samples were visualized by using two different SEM microscopes: a JEOL-JSM 6100 microscope attached to an EDAX unit (Link EDAX Analytical model AN10/85S) and a JEOL-JSM 6060 LV microscope.

Slide preparation. Standard microscope slides were first ground (Logitech LP30 apparatus) to obtain perfectly flat ($\pm$ 2 μm) and parallel surfaces. Carborundum paste (Silicon Carbide) of grade 600 (each particle 9 μm in diameter) was used as a grinding liquid. Devcon "2-ton" epoxy resin was smeared on the frosted side of the prepared slide. Calcite crystals were spread on the smooth side of the slide. The sample was pressed down using more resin. The prepared slide was placed on a hot plate at 90°C for 30 min to lower the viscosity of the resin, allowing the crystals to become immersed. After cooling, the prepared slide containing the resin and calcite crystals was ground (Petro-thin thin-sectioning system; Buehler) from an initial thickness of 993 μm (including the slide, resin, and calcite crystals) to a final thickness of 100 μm. This procedure resulted in crystals of different thicknesses. However, due to the crystals being embedded in the resin it was not possible to determine the thickness of ground crystals.

To produce limestone slides, the limestone pieces were cut into 1-cm-thick rectangles (area of 1,096.41 mm^2). Fixation of the different limestone rectangles onto the glass microscope slides followed the same procedure as that used for the preparation of the slides bearing crystals. The surfaces of these samples were prepared as described for the thin sections, producing a polished surface, but no resin was applied, permitting surface pore spaces to be left hollow and permitting access to the interior pore structure of the stone. The final thickness of the limestone slides was 500 μm.

Application of biocalcifiers to limestone. One milliliter of an overnight culture of isolates F2, D2, and B14 incubated at 30°C in B4 liquid medium (9) was spread on the surface of different limestone slides (500 μm thick). The controls consisted of uninoculated limestone slides and limestone slides inoculated with B4 liquid medium alone. All the limestone slides were placed on filter paper (Whatman No. 1; Fisher Scientific, United Kingdom) (90 mm in diameter) in a petri dish and incubated for 3 weeks at 30°C. The filter papers were wetted with 1 ml of sterile dH_2O at regular intervals to moisten the stone. This was repeated as many times as required. The stone slides, both inoculated and uninoculated, were also treated with 500 μl of B4 medium once a week.

Staining techniques. (i) Crystals mounted on glass slides. Bacteria around and inside carbonate crystals were detected using Loeffler's methylene blue stain (Fisher Scientific, United Kingdom). Carbonate crystals mounted on glass microscope slides were stained with Loeffler's methylene blue (methylene blue, 3 g; potassium hydroxide, 10 mg; 95% ethanol; 300 ml per liter of dH_2O) for 1 min, rinsed with dH_2O, dried by blotting, and observed with white light by the use of ×1,000 magnification and immersion oil.

Polysaccharides associated with carbonate crystals were detected using alcian blue–periodic acid-Schiff stain (PAS). Crystals on glass microscope slides were stained with alcian blue-PAS (Fisher Scientific, United Kingdom) by applying 100% ethanol for 5 min, rinsing with dH_2O for 5 min, applying alcian blue for 5 min followed by 1% Schiff's reagent for 5 min, and, finally, rinsing with dH_2O for 5 min.

(ii) Limestone slides. In order to determine the distribution of bacteria, CTC (5-cyano-2,3-ditolyl tetrazolium chloride; Polysciences Inc.), which stains active respiring bacteria, was used to visualize the bacteria to show where they were in the crystals and on the limestone slide. Before the control preparation was stained, stone slides were fixed for 30 min with 4% paraformaldehyde–2× phosphate-buffered saline. The slides were flooded with freshly prepared 1.5 mM (final concentration) CTC solution. The stain solution was left to react overnight at room temperature in the dark, and the slides were stored at −20°C for 30 min to stop the reaction. Before visualization with a confocal scanning laser microscope (LSM 510 Meta; Carl Zeiss), the slides were covered with a coverslip and fixed with DPX mountant (BDH, Poole, United Kingdom). The stain was excited at 453 nm by the use of a 590-nm long-pass filter. The slides were visualized using ×400 magnification and immersion oil.

Image analysis. Before the different limestone slides were stained with CTC, five different areas of the limestone slides were photographed using a stereoscopic microscope (Wild M4A TYP 376788; Heerbrugg, Switzerland) before and after application of the biocalcifying B14, D2, and F2 isolates. MapInfo software (MapInfo Professional v. 6.0; MapInfo Corporation) was used to measure the area of pore spaces on the stone slide before and after treatment. Mapping allowed visual identification and quantification of individual pore spaces. The quantitative data were used to calculate the descriptive statistics (Minitab statistical software; Minitab release 13.1, 2000) that were used to identify pore space reduction results.

Image analysis packages have been used since the late 1980s for classifying and quantifying pore space in rocks (see, e.g., references 31 and 43 for later examples of analyses performed using SEM imagery). In this study, however, the identification and quantification of the open pores on the surface of the samples was carried out by an observer. The observer identified and then digitized the open pore data shown on screen. The software package was then used to classify each

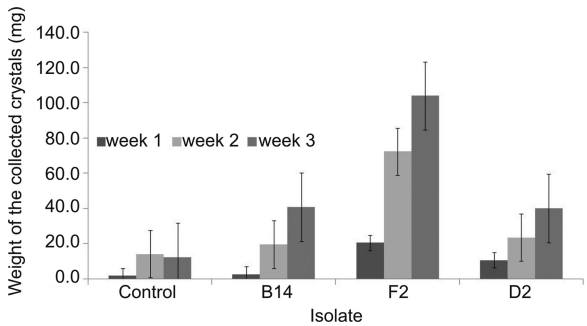

FIG. 1. Calcium carbonate crystals precipitated by isolates B14, F2, and D2 at 30°C during 3 weeks in B4 liquid medium. Error bars, standard errors of the means.

of the digitized polygons as a single entity and and to calculate and store information about that entity such as area and perimeter length. The observer then continued to identify and digitize the data for the other open pores in the image. This meant that no automatic classification software was used in identifying and mapping the open pores on the surface of the sample and that the whole system relied on observer skill at identification and digitizing. This more labor-intensive method of image analysis was used to ensure that there was a consistent, observer-based set of decisions made concerning pore space identification. Although this method had not been applied to this type of experimental situation before, it was thought that having the observer perform as the classifier would reduce potential errors that an automatic identification system might introduce. Additionally, each area of the sample where an image was taken was then relocated after the application of biocalcifiers. Relocation was possible because each sample had a series of control points on its surface that enabled a given area to be imaged before and after the experiment. The same observer then carried out the same identification and digitizing procedure on the altered surface of the sample. Using this method, it was possible to locate and map changes (if any) in the extent of specific open pore spaces from before and after treatment. This means that all data regarding changes in the extent of open pore spaces refer to changes mapped for the same open pores through time rather than to measurements of average changes determined using different open pores sampled before and after the experiment.

Cell viability inside carbonate crystals. (i) Culture preparation and crystal collection. Carbonate crystals precipitated by isolates D2, B14, and F2 were collected after 3 weeks of incubation and placed into different sterilized 1-ml Eppendorf tubes. For analyses of cell viability, carbonate crystals were treated as follows: 50-ml liquid cultures, grown in 100-ml conical flasks, were poured into individual 50-ml Fisherbrand Falcon tubes (Fisher Scientific, United Kingdom) and centrifuged for 10 min at 2,500 rpm (Camlab alc 4232 centrifuge). The supernatant was then removed. In order to maximize recovery of crystals, the conical flasks were washed twice with 15 ml of sterile dH_2O and this suspension was also centrifuged. The recovered crystals were washed with 5 ml of 70% ethanol for 20 min, and then the crystal suspensions were recentrifuged and the supernatant was discarded. The collected crystals were distributed equally into sterile 1-ml Eppendorf tubes, sealed, and stored at room temperature for up to 330 days.

(ii) Release of bacteria from carbonate crystals to study cell viability. The different sealed samples were analyzed in triplicate after 5, 13, 36, 76, and 330 days for cell viability determinations. The collected crystals were transferred into an agate pestle and mortar that were washed and sterilized at 121°C for 15 min. The crystals were crushed until they were reduced to a fine powder. The carbonate powder was resuspended in 1 ml of sterile dH_2O and serially diluted in 9 ml of sterile quarter-strength Ringer's solution. Ten milliters of each of four consecutive 10-fold dilutions (10^{-1} to 10^{-4}) for each of the different isolates was filtered through a microfunnel (Life Science, United Kingdom) (0.2-μm-pore-diameter white gridded SP filter unit with support membrane) placed on an electrical filtration unit (Gelman Sciences) (three-filter unit). After filtration, the membranes were placed onto plate count agar (Oxoid, United Kingdom) medium in 45-mm-diameter petri dishes and incubated at 30°C overnight. Colonies were counted by placement of the membrane on the petri dish lid and observation under conditions of white light and ×400 magnification. Uncrushed crystals were used as a control.

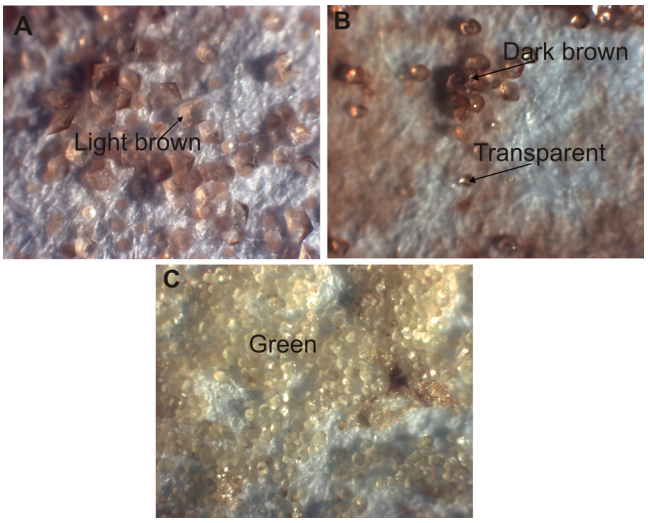

FIG. 2. Carbonate crystals precipitated by isolates B14 (light brown) (A), D2 (dark brown and transparent) (B), and F2 (greenish and brown) (C) at 20°C in B4 liquid medium after a 3-week incubation. Magnification, ×600.

RESULTS

Carbonate crystal precipitation. No carbonate crystals were precipitated in the controls. Isolates B14, D2, and F2 were able to precipitate carbonate crystals at 30°C over the 3-week period of incubation (Fig. 1). The carbonate crystals precipitated were either clear (transparent) or colored, usually brown, covering a wide range of shades from light to dark. F2 was the only isolate that precipitated light-green carbonate crystals. Figure 2 shows carbonate crystals precipitated by the three isolates.

XRD analysis of the carbonate crystals precipitated by isolates D2, F2, and B14 during the third week of incubation identified the crystals as representing calcite and vaterite. Calcite was the main polymorph precipitated by isolates D2 and F2. However, vaterite was the main polymorph precipitated by isolate B14 (Fig. 3). SEM revealed that the carbonate crystals precipitated by the different isolates had a spheroidal shape and that they were mostly covered by bacteria (Fig. 4).

Histological staining of carbonate crystals. Thick sections of the crystals precipitated by isolate F2 showed that bacteria surrounded the precipitated carbonate crystals (Fig. 5A). Thin sections of precipitated crystals indicated that bacteria were not only at the center of the precipitated carbonate crystals but were also evenly distributed between the inner core and the outer surface of the precipitated crystals, as well as being present on the outer surface (Fig. 5B and C). Thick sections of the carbonate crystals precipitated by B14 stained with alcian blue-PAS revealed that a mass of neutral polysaccharides was located in the center of the crystals; this mass was surrounded by a nonstaining, presumably inorganic layer, but some polysaccharides were also stained around this inorganic layer (Fig. 5D). Thin sections revealed that along with the neutral polysaccharides there were also acid polysaccharides and that both types of polysaccharides were distributed in a well-organized manner (Fig. 5E and F). Crystals that had become attached to each other to form sheet-like structures were stained with alcian blue-PAS; this stain revealed that the crystals were also

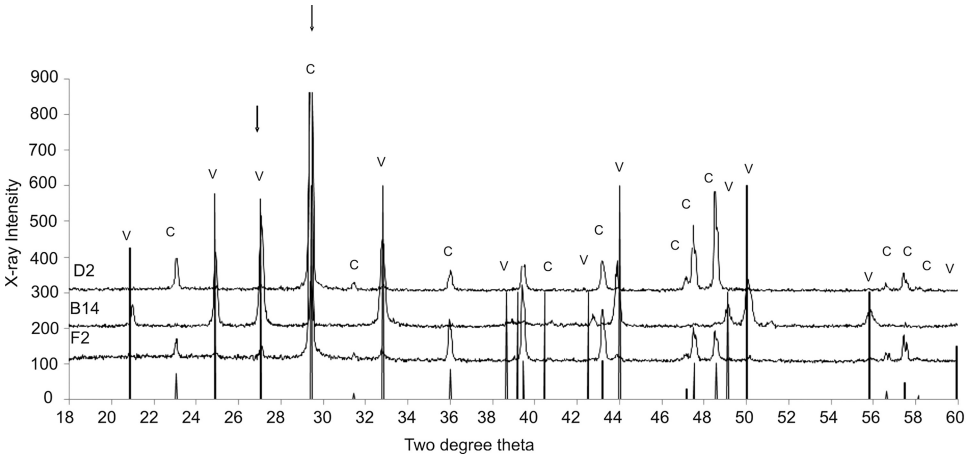

FIG. 3. XRD chart of the carbonate crystals precipitated by isolates B14, D2, and F2 in B4 liquid medium at 30°C. V, vaterite; C, calcite. Arrows indicate the main vaterite and calcite peaks.

bound by a mixture of acid and neutral polysaccharides (Fig. 5G).

Biocalcifiers on stone. (i) Changes in open pore size after biocalcifying treatment. Observation of the surface of the limestone slides under the light microscope showed that the crystals were preferentially deposited around and inside the open pore spaces (Fig. 6 and 7). Treatment with B14 medium alone reduced the area of open pore space by 19%, but when the different isolates were examined this reduction in area was much greater with isolate B14 (with a reduction of 43%), isolate F2 (reduction of 46%), and isolate D2 (reduction of 49%) (Table 1); this illustrates that the application of the isolates resulted in at least 20% more of the open pore space being filled than the application of the medium alone.

(ii) Fluorescence staining of crystals. Fluorescence microscopy with CTC revealed few live bacteria on the stone after 21 days of incubation at 30°C, and the majority of bacteria seemed to be intimately linked with the carbonate crystals. The stain did not react with the stone material, but it did produce strong fluorescence with the bacterially precipitated carbonate crystals. Carbonate crystals produced abiotically were also visualized with CTC, but the fluorescence was less intense than with the bacterially precipitated crystals and the images were not so clear (Fig. 8).

Cell viability inside carbonate crystals. Stored crystals were crushed and assessed at intervals for the presence of bacteria that were able to form viable, visible colonies on plate count agar. The numbers of viable cells per milligram of crystal decreased with time (Fig. 9). No bacterial growth occurred on membranes onto which uncrushed crystals (controls) were deposited and incubated under the same conditions as those used for crushed crystals. B14 was the isolate whose crystals initially had the highest number of viable cells per milligram; the lowest number was found with isolate F2. The number of viable cells per milligram from crushed crystals precipitated by isolate D2 could not be counted at the first sampling event (day 5), because the cells were strongly aggregated. Bacterial viability decreased rapidly after 13 days irrespective of the type of isolate. Although there were fluctuations in the counts determined between days 36 and 76, bacterial viability tended to decrease with time. We also noted that cells from 5-day-old crystals, when incubated at 30°C, produced visible colonies in 24 h, whereas cells from older crystals required 3 to 4 days of incubation to form colonies that were visible only when viewed under a light microscope (×400 magnification). After 330 days, at the end of the experiment, no viable cells were found in the carbonate crystals precipitated by isolates B14 and F2, and the few viable cells (approximately 9 cells/mg) from the carbonate crystals precipitated by isolate D2 were counted.

DISCUSSION

Carbonate crystal precipitation. The isolates studied were able to precipitate carbonates under the chosen experimental conditions at 30°C, and the amount of precipitation increased with incubation time. The optimum temperature for bacterial carbonate precipitation has been established to be in a range between 22°C and 32°C (24, 48, 52, 55). The relationship between the extent of carbonate precipitation and incubation time could not be related to an increase in biomass. Zamarreño et al. (63) observed that although there was an initial increase in cell concentrations, after 21 days of incubation at 30°C in liquid B4 medium no significant increase was detected; i.e., counts per milliliter for isolate B14 changed from 3.40×10^2 to 6.4×10^2. However, crystal production could be related to an increase in the binding ability of the cell occurring with increasing age. Shuttleworth and Unz (58) observed that the ability of *Thiothrix* sp. strain A1 to bind to Ni or Zn was higher in 2- and 5-day-old cells than in 1-day-old cells.

The three freshwater isolates B14, D2, and F2 precipitated both calcite and vaterite. There is uncertainty about what causes bacteria to precipitate different carbonate polymorphs. Although the composition of the growth medium has been seen to have some effect on the type of carbonate precipitated (48, 50, 51), the particular bacterial species used has an important influence on the type of carbonate precipitated, as found for *Halomonas eurihalina* (49) and *Nesterenkonia halobia* (53). Cacchio et al. (13) demonstrated that, with respect to carbonate precipitation on B4 agar by *Kocuria*, *Acinetobacter*, *Bacillus*, and *Renibacterium* spp., only *Kocuria* spp. were able to

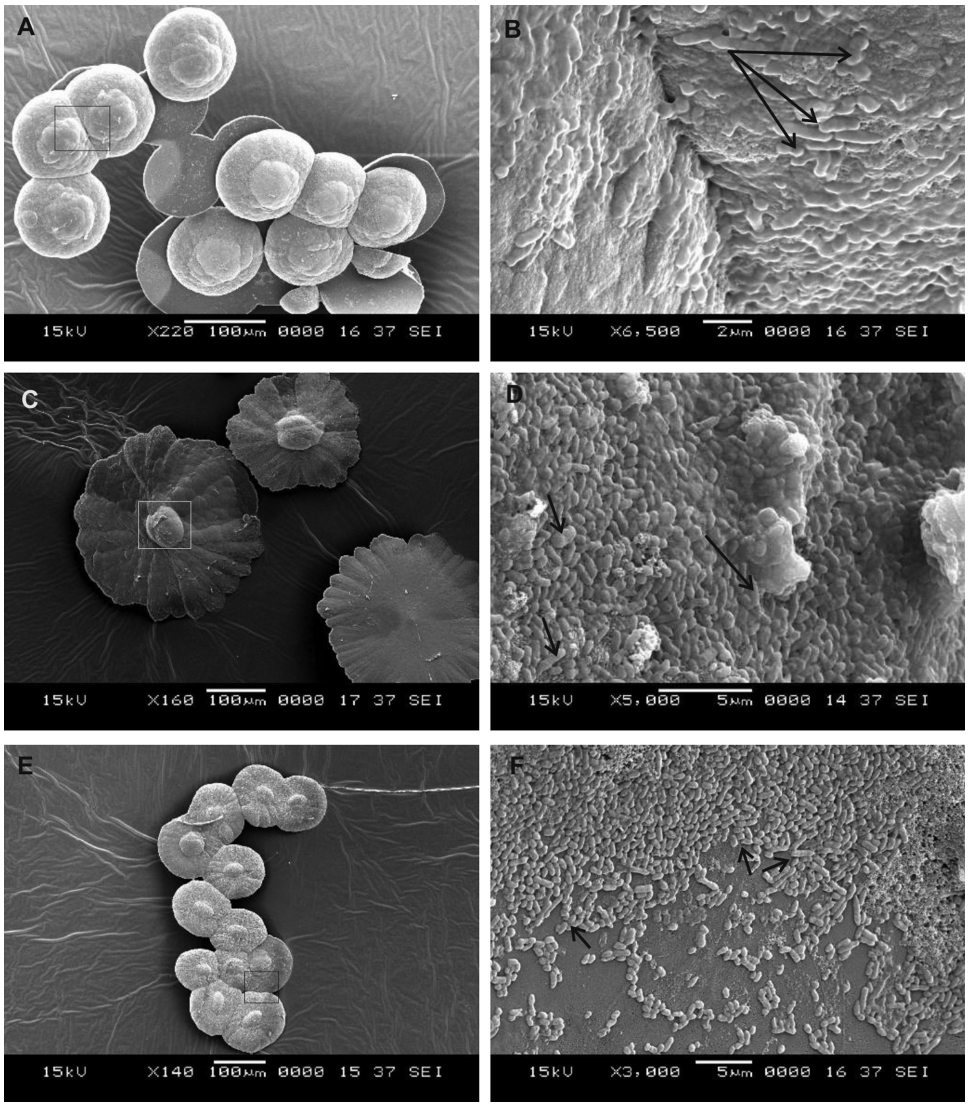

FIG. 4. Carbonate crystals precipitated by isolates F2 (A and B), B14 (C and D), and D2 (E and F) at 30°C in B4 liquid medium. All the different carbonate crystals had a spheroidal shape and were covered by bacteria (see arrows and arrowhead in panels B, D, and F).

precipitate vaterite. However, work on bioconsolidation of stone by *Myxococcus xanthus* (56) and on bioconsolidation of cement by *B. sphaericus* (19) showed that both bacterial species were able to precipitate calcite and vaterite.

The precipitation of various types of carbonate crystals by different bacteria has been suggested to be related to the optimal growth conditions required (18). Thus, unstable carbonate polymorphs, such as vaterite and aragonite, may be precipitated under unfavorable growth conditions and the most stable polymorphs, calcite and dolomite, are produced under optimal growth conditions. However, Braissant et al. (10) related the precipitation of the different carbonate polymorphs to bacterially precipitated EPS composition and concentration.

The coloration of the bacterially precipitated carbonate crystals is not a unique feature of the isolates under study. Hammes et al. (30) found that crystals precipitated by ureolytic bacteria isolated from different sources presented a wide range of brown shades. Bacteria have also been associated with the formation in caves of calcareous structures such as speleothems and stalactites (5). In speleothems, a variety of colors may be present, depending on the ions that can substitute for the Ca^{2+} ions: e.g., Fe^{2+} (pink color of Greenbrier limestone in Randolph Country, WV), Ni^{2+} (green aragonites in Timpanogos Cave, UT), and Cu^{2+} (blue-green in Grotte Bleue, Italy) (62). However, White (62) found that the yellow, tan, orange, and brown colors observed in some calcareous speleothems were not related to iron oxides but to organic pigments such as humic and fulvic acids. Northrup et al. (36) and Cacchio et al. (13) found that bacteria isolated from speleothems were able to precipitate carbonate crystals in the laboratory. The observations in our study, in which different colored carbonate crystals were precipitated by the isolates studied, suggest that the color of speleothems could also be due to bacteria involved in their formation.

Internal composition of the carbonate crystals. When carbonate crystals precipitated by isolates B14, D2, and F2 were

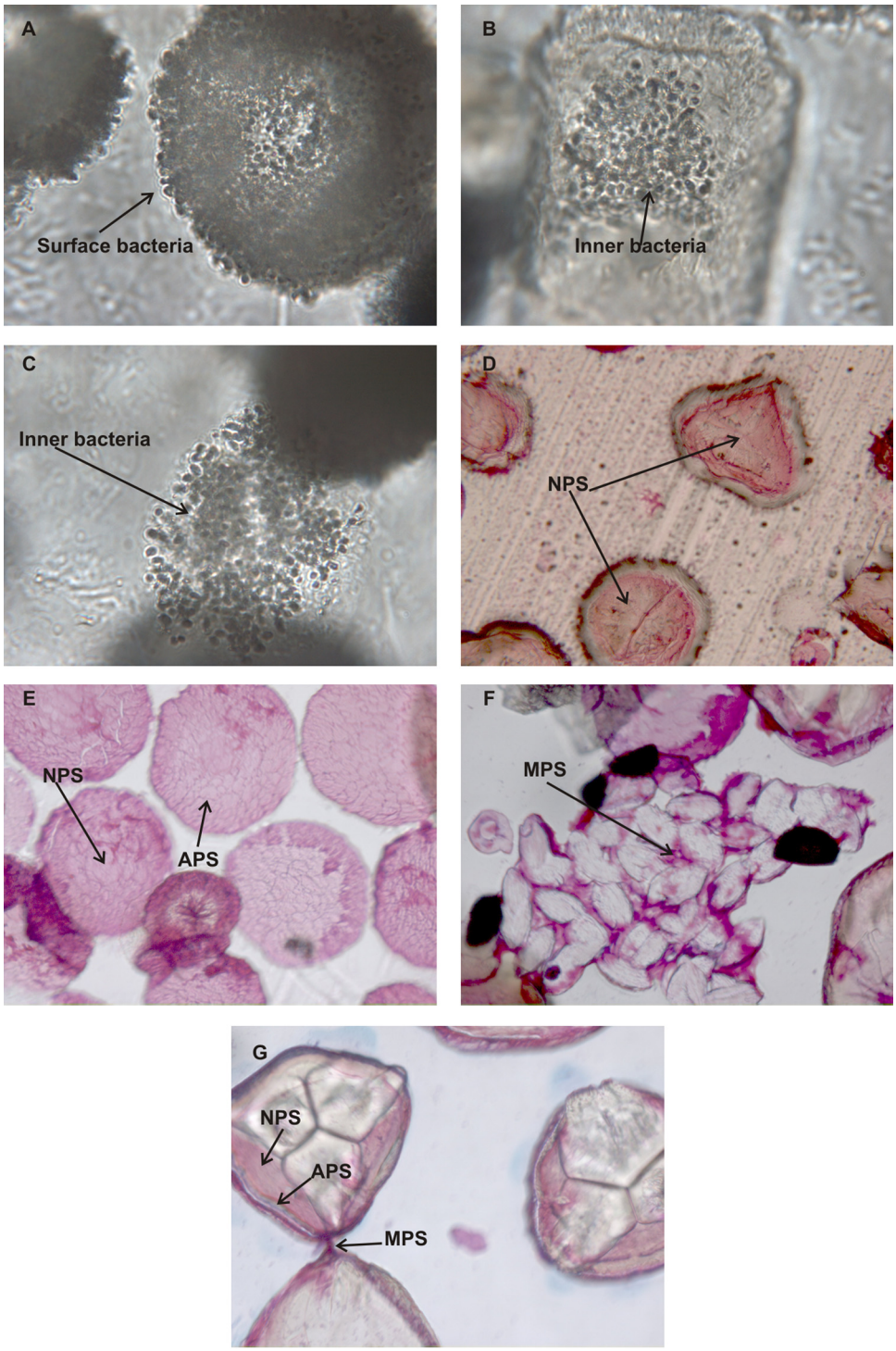

FIG. 5. Light microscopy micrographs of sections of carbonate crystals precipitated by F2 and B14 in B4 liquid medium at 30°C. Crystals stained with Loeffler's methylene blue. (A) Thick section of a carbonate crystal showing surrounding bacteria. (B) Thin section showing bacteria forming part of the core of the precipitated carbonate crystals. (C) Thin section showing bacteria distributed in the inner matrix of the carbonate crystals in immersion oil (magnification, ×1,000). Bacteria were visualized as dark dots over a light-blue background. Crystals were stained with alcian blue-PAS. (D) Thick section showing neutral polysaccharides in the interior of the carbonate crystals surrounded by an inorganic phase, with associated polysaccharides on the outer surface. (E) Thin sections showing the distribution of neutral and acid polysaccharides inside the carbonate crystals (magnification, ×200). Unground carbonate crystals are shown. (F) Sheet-like structures precipitated by isolate F2. The different intensities in the magenta color indicate that a mixture of acid and neutral polysaccharides bound the crystals to each other. (G) Carbonate crystals precipitated by isolate B14 joined by a mixture of acid and neutral polysaccharides. Magnification, ×200. NPS, neutral polysaccharides; APS, acid polysaccharides; MPS, mixture of neutral and acid polysaccharides.

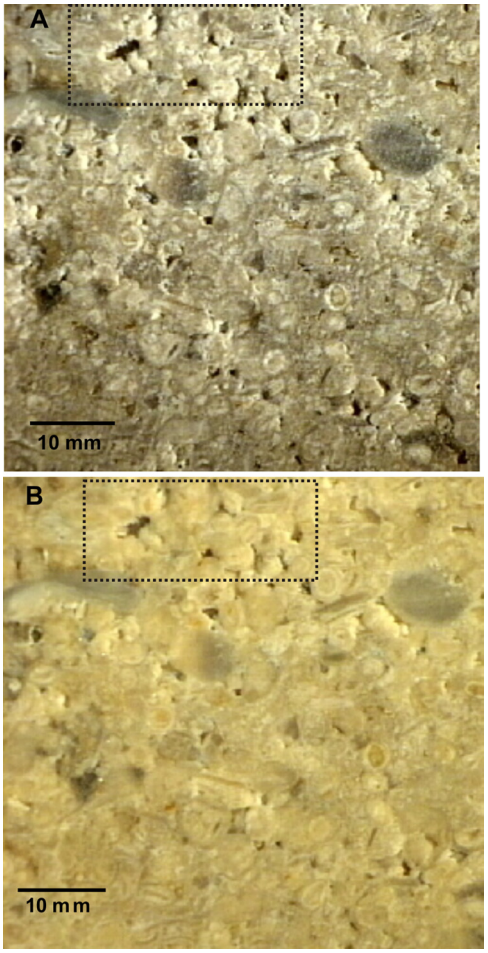

FIG. 6. Isolate D2 on limestone before treatment (A) and after bacterial biocalcifying treatment for 21 days of incubation at 30°C (B). The rectangles in each panel allow visual comparison of pore size changes before and after treatment. The quantitative analysis of actual changes in pore size was done using MapInfo image analysis software, and the details of the treatment are given in Table 1.

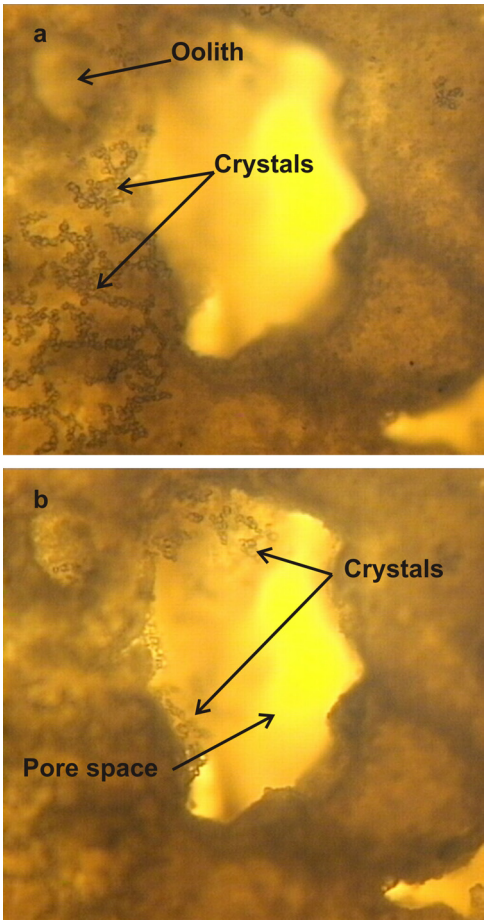

FIG. 7. Typical carbonate crystal precipitation by isolate D2 on limestone after 3 weeks of incubation in B4 liquid medium at 30°C. The two images were taken at different focal planes of the same pore, showing carbonate crystals deposited on top of ooliths (a) and inside the pore space (b). Magnification, ×200.

ground and stained with alcian blue-PAS, a mass of neutral polysaccharides in which bacteria (as visualized separately by the use of Loeffler's methylene blue stain) were embedded inside the crystal structures was revealed. EPS not only were found inside the carbonate crystal but also facilitated crystal-to-crystal attachment to form sheet-like structures. These findings are in agreement with previous research by Geesey and Jang (26), Buczynski and Chafetz (11), and Van Lith et al. (60), whose results showed that EPS play an important role in the formation of carbonate crystals not only by providing nucleation sites but also by attaching small crystals to each other to increase the size of the bioliths.

The viability of the bacteria entrapped inside the carbonate crystals has long been a subject of discussion by Greenfield (28), Krumbein (32), and Parsek and Singh (40). In our experiments, we found large numbers of bacterial cells in 5-day-old crystals but fewer as time progressed. The conflicting results presented in previous studies may have been due to two different factors, because our results suggest that both the age of the crystals and the type of bacterial species appear to be important, since the counts of bacteria inside the carbonate crystals declined with time and the bacteria in only one of the isolates under study, D2, were able to survive for nearly a year, although the number of surviving cells, i.e., 9 cells/mg, was negligible. This result also contrasts with the report of Appanna et al. (2), who suggested that, by becoming entrapped inside carbonate crystals, bacteria could survive for long peri-

TABLE 1. Change in the size of the pore space before and after treatment with B4 medium alone, B14, D2, and F2 after 21 days at 30°C

Treatment	No. of pore spaces analyzed	Area of pore space (μm^2)				% Reduction
		Before treatment		After treatment		
		Mean	Standard error of the mean	Mean	Standard error of the mean	
Medium alone	264	3.43	0.232	2.77	0.226	19
B14	479	2.70	0.220	1.73	0.431	43
D2	211	3.70	0.255	2.35	0.299	46
F2	442	3.97	0.377	2.06	0.279	49

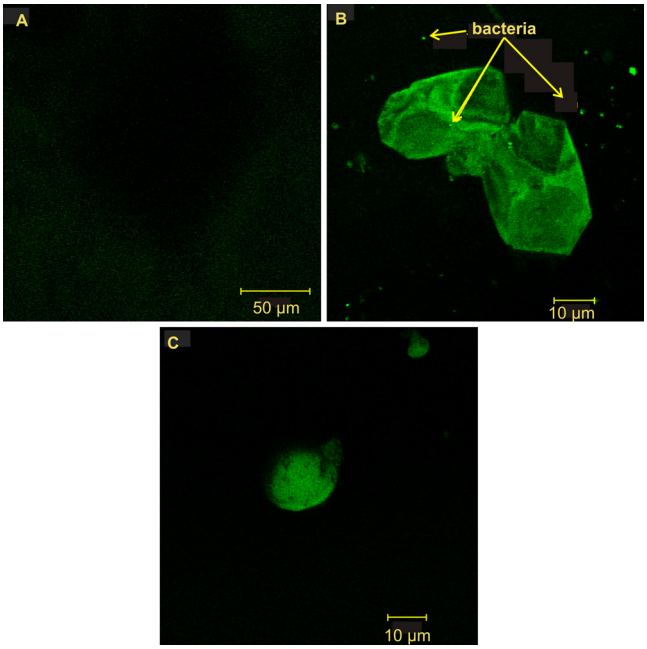

FIG. 8. Fluorescence microscopy after staining with CTC. (A) limestone control; (B) limestone inoculated with isolate F2; (C) abiotic crystal. (A) The CTC stain did not react with the limestone slide. (B) Crystals (large fluorescent objects) precipitated by F2 after 21 days at 30°C. The arrows indicate groups of bacterial cells. (C) Abiotic carbonate crystals produced chemically in B4 medium by increase of pH to 9.5. CTC stained the abiotic crystals less strongly than the biological means described for panel B.

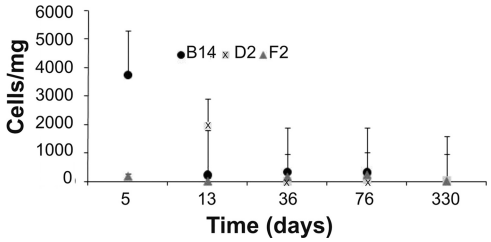

FIG. 9. Bacterial survival at room temperature inside carbonate crystals precipitated by isolates B14, D2, and F2. On day 5, the bacterial colonies grown from D2 crystals were found to be strongly aggregated.

ods of time, protecting themselves from an environment oversaturated with Ca^{2+} ions. Submersion of the carbonate crystal in 70% ethanol for 20 min did not kill the encased bacteria. If bacteria die with the passage of time inside the crystals, why do bacteria form them? This question may have two possible answers, namely, that bacteria cannot avoid crystal formation (passive formation) or that crystal formation is an active process that protects bacteria, for a short period of time, from an environment with excess Ca^{2+} ions that can be detrimental.

Application of biocalcifiers on limestone slides. Fluorescence microscopy revealed that most of the biocalcifiers applied to the stone surface were forming crystals and that few remained as single cells after 21 days of incubation at 30°C. Le Métayer-Levrel et al. (33) observed that *Bacillus* spp. applied as biocalcifiers on limestone were still viable and producing carbonates even 1 year after the initial application and that this activity did not allow the establishment of other bacterial species that could be detrimental to stone.

The size of pore spaces (porosity) between the calcite crystals in natural stone is considered to be one of its most important features, since it is in these spaces that the products of pollution and water tend to accumulate and react with the stone components (16). These chemical reactions produce changes in physical properties such as increases in porosity, leading to weakening of the structure and consequently to accelerated decay (1).

Treatment of the limestone slides with biocalcifying bacteria seemed to reduce the area of open pore space by at least 20% more than the application of isolate medium alone. Rodriguez-Navarro et al. (56) used mercury intrusion porosimetry (MIP) to study changes in pore size due to biocalcification by *M. xanthus*; however, the MIP method was not sensitive enough to detect the small changes produced by bacterially precipitated calcium carbonates. An important issue with using "standard" methods for evaluating changes in porosity in relation to calcifying bacteria is that these methods have often been developed to assess the bulk properties of samples. Calcifying bacteria act upon the surface of a sample and then penetrate into the pore structure in the rock. Methods such as MIP require a sample of rock that is much larger than just the surface where bacteria will initially act. Measurement of pore space by the use of the method named above, for example, involves forcing mercury under applied pressure into the pore spaces in the rock but only into those pore spaces that are connected. This means that the volume of space within the whole sample is measured, representing a bulk property of the whole sample and one that is unlikely to be greatly influenced by the development of a potentially thin and fragile film of biocalcifying bacteria on the sample surface. Similarly, a method that forces mercury into pore spaces under applied pressure may destroy the surface structures that could prevent ingress of material. The depth of penetration of biocalcifying bacteria into a rock could be analyzed using fracture samples and identifying bacteria through staining or via SEM observations. Likewise, impregnation with resin followed by thin-section analysis of a vertical section of stone could provide similar information on the depth of penetration of biocalcifying bacteria. Such information is important in assessing the depth of penetration of such bacteria, but these methods do not necessarily provide information on the relationship between open pore spaces and bacteria on the surface of the sample. They do not provide information on the spatial extent of the relationship between open pore space and bacterial growth. The novel method of using observer-defined mapping of the same open pore spaces before and after treatment with biocalcifying bacteria does provide that information. The evidence from this analysis suggests that biocalcifying bacteria seem to preferentially fill pore spaces but that they do not eliminate these spaces, an effect that would have serious implications for water movement and the physical behavior of the stone (6). Any consolidant technique has the potential to cause this damaging effect, and this has long been a concern of stone conservators.

The 19% reduction in open pore space that medium alone produces could illustrate that the "roughened" or depressed element of the surface would be filled regardless of what is

applied to the surface. The additional 20% filled by the bacteria, it could be argued, merely reflects preferential growth in such "roughened" areas. On a flat surface, as often found on stonework, open pore spaces are a part, and an important part, of the roughened surface, so evidence that both medium and bacteria will preferentially flow or grow at these locations does not negate the observations made concerning the loss of open pore space. The observations may require qualification as rougher, more "natural" rock surfaces are studied, but at present all that can be stated from these experiments is that open pore spaces on a flat surface provide both a sink for medium and a growth niche for bacteria. Whether this statement can also be made regarding other "roughness" elements cannot be clarified from this set of experiments.

Concluding remarks. Our research has extended understanding of the composition of bacterially precipitated carbonate crystals and evaluated the use of freshwater bacteria as a stone bioconsolidant. Carbonate crystals were treated with histological stains for the first time to reveal that they were formed around a mass of polysaccharides in which bacteria were embedded. Sheet-like structures were formed when small crystals were bound together by EPS. Both histological and fluorescence microscopy revealed that the outer coating of the precipitated carbonate crystal was formed by bacteria. CTC staining showed that active bacteria were rarely present as single cells or/and aggregates but were an integral part of the newly precipitated carbonate crystals.

Investigation of bacterial viability inside carbonate crystals revealed a short (about 2-week) life span, so dissolution of carbonate crystals is unlikely to result in uncontrolled bacterial growth. A further significant finding of the work is that a reduction, rather than a blockage, of pore space occurs during consolidation. Taken together, the results of this research provide some reassurance to conservators that biocalcification by bacteria could safely be applied in a restoration strategy for building stone conservation.

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