

# Calcium Carbonate Mineralization: Involvement of Extracellular Polymeric Materials Isolated from Calcifying Bacteria

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**Abstract:** This study highlights the role of specific outer bacterial structures, such as the glycocalyx, in calcium carbonate crystallization *in vitro*. We describe the formation of calcite crystals by extracellular polymeric materials, such as exopolysaccharides (EPS) and capsular polysaccharides (CPS) isolated from *Bacillus firmus* and *Nocardia calcarea*. Organic matrices were isolated from calcifying bacteria grown on synthetic medium—in the presence or absence of calcium ions—and their effect on calcite precipitation was assessed. Scanning electron microscopy observations and energy dispersive X-ray spectrometry analysis showed that CPS and EPS fractions were involved in calcium carbonate precipitation, not only serving as nucleation sites but also through a direct role in crystal formation. The utilization of different synthetic media, with and without addition of calcium ions, influenced the biofilm production and protein profile of extracellular polymeric materials. Proteins of CPS fractions with a molecular mass between 25 and 70 kDa were overexpressed when calcium ions were present in the medium. This higher level of protein synthesis could be related to the active process of bioprecipitation.

**Key words:** calcite precipitation, EPS, CPS, biofilm, calcifying bacteria, historic monument protection

## INTRODUCTION

Microorganisms influence the formation of different minerals, such as carbonates, sulfates, and silicates (González-Muñoz et al., 2010). Many different bacterial species precipitate carbonate in alkaline environments rich in  $\text{Ca}^{2+}$  ions. Numerous authors have described the capability of bacteria to precipitate carbonate in both natural habitats and laboratory culture. Various mechanisms, which could induce precipitation by bacteria in natural habitats, have been proposed (Erlich, 1996; Rivadeneyra et al., 2004). However, the precise roles of bacteria and bacterial activities in the process of carbonate crystallization remain unclear, although they seem to fall categorically into three different yet related tracks.

As a first hypothesis, mineralization may be seen as a by-product of microbial metabolism involving either autotrophic or heterotrophic pathways (Rivadeneyra et al., 1994; Douglas & Beveridge, 1998; Castanier et al., 1999; Lian et al., 2006). In these passive processes, reactions, such as the enzymatic hydrolysis of urea or the dissimilatory reduction of nitrate and sulfate, cause an increase in pH that shifts the bicarbonate-carbonate equilibrium toward the production of more  $\text{CO}_3^{2-}$  and ultimately the precipitation of  $\text{CaCO}_3$ , if free  $\text{Ca}^{2+}$  is present. As an alternative hypothesis,  $\text{CaCO}_3$  precipitation is controlled by intracellular calcium metabolism rather than changing  $\text{CO}_3^{2-}$  concentration (Anderson et al., 1992; McConnaughey & Whelan, 1997; Hammes & Verstraete, 2002).

As a second hypothesis, carbonate nucleation takes place on the cell wall, either due to the ion exchange

through the cell membrane (i.e., an active process proposed by Castanier et al., 2000) following some still poorly known mechanisms, or due to the support of negatively charged specific cell wall functional groups that adsorb divalent cations, such as  $\text{Ca}^{2+}$  (Schultze-Lam et al., 1996; Rivadeneyra et al., 1998). These  $\text{Ca}^{2+}$ -cell wall interactions modify the cell wall, allowing interactions 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 (Ferrer et al., 1988; Rivadeneyra et al., 1996, 1998; Zamarreño et al., 2009).

The third hypothesis involves extracellular macromolecules. The extracellular polymeric materials [i.e., exopolysaccharides (EPS) and capsular polysaccharides (CPS)] produced by bacteria form a large class of polymers implicated in various processes such as the formation of structure and architecture of biofilm matrices and calcium carbonate precipitation (Ercole et al., 2007; Decho, 2009; Ditttrich & Sibling, 2010). Extracellular polymeric materials consist of various organic substances, including mostly polysaccharides and proteins, but also nucleic acids, lipids, and uronic acid (Nichols & Nichols, 2008). Both CPS and EPS produced by microorganisms may be tightly bound to the cell (cell attached or capsular), loosely adherent to cells (slime type, free, or released) or in the form of free dissolved matter (Nielsen & Jahn, 1999; Bhaskar & Bhosle, 2005). These biopolymers can trap calcium ions at a given pH or serve as growth modifiers controlling crystallization and influencing the polymorphic development of  $\text{CaCO}_3$  during mineralization, such as observed in cyanobacteria (Kawaguchi & Decho, 2002; Braissant et al., 2003; Dupraz et al.,

2009). Hammes et al. (2003) suggested that specific proteins present in biological extracellular polymeric materials may cause the formation of different and polymorph  $\text{CaCO}_3$  bioliths.

Different mechanisms, which could induce precipitation in natural habitats by bacteria, have been proposed and active and passive roles of bacteria in these processes have been suggested (Erlich, 1996; Morita, 1980; Novitsky, 1981). However, in many cases their precise role in carbonate formation is not well known.

We here describe the *in vitro* formation of calcite crystals by extracellular polymeric materials (EPS and CPS) isolated from calcifying-bacterial strains and provide evidence showing that some protein components present in these fractions could be involved in the bioprecipitation process. Bacterially induced carbonate mineralization has been proposed as an environmentally friendly method to protect decayed ornamental carbonate stone (Le Mêtayer-Levrel et al., 1999).

Biocalcification by bacteria is an emerging restoration technique that is still being developed and requires further research. However, the production of acid substances, the development of colored spots by microbial metabolism, or bacterial survival within the carbonate crystal could have serious implications in restoration techniques. It has already been demonstrated that uncontrolled bacterial growth can damage stone (Papida et al., 2000; Warscheid & Braams, 2000; Zanardini et al., 2002; Perry et al., 2003; Sarró et al., 2006; Zamarreño et al., 2009). To overcome these problems, genes and proteins involved in the mineralization process should be isolated from calcifying bacteria and identified in order to utilize proteins without living cells.

In this study, we investigated bacterially mediated precipitation and, specifically, how the extracellular polymeric materials associated with bacterial biofilms affect the process of  $\text{CaCO}_3$  precipitation. In particular, we focused our research on two bacteria isolated from an Italian karst cave: *Bacillus firmus* and *Nocardia calcaea* because (a) in a previous work we demonstrated the involvement of EPS and CPS isolated from *Bacillus* in calcite crystal formation and (b) *N. calcaea* produce mucoid colonies that precipitate very quickly, within 30 h, calcite crystals coated with a thick "mucilage" (Macilenti, 2002; Bozzelli, 2011).

The aim of the present work was: (1) to isolate EPS and CPS from *B. firmus* and *N. calcaea* grown on synthetic medium, in the presence and absence of calcium ions, (2) to show that calcium ions promote protein synthesis in extracellular polymeric materials and influence biofilm production, and (3) to investigate protein involvement in the calcifying process.

## MATERIALS AND METHODS

### Bacterial Strains and Culture Media

The calcifying-bacterial strains utilized in this study were isolated from Stiffe cave near L'Aquila (Central Italy). In a previous study, the bacteria were classified as *B. firmus* and *N. calcaea* (Macilenti, 2002; Cacchio et al., 2003).

Bacterial strains were maintained on slants of B4M medium (Bouquet et al., 1973) or the modified form B4Mmod. This last contains sodium acetate (2.3 g/L) instead of calcium acetate (2.8 g/L) present in B4M.

### Growth of Bacterial Biofilm and Colorimetric Assay

Biofilm formation was tested to examine the influence of calcium ions on biofilm formation by determining the ability of *B. firmus* and *N. calcaea* to adhere to the wells of 96-well polypropylene dishes (O'Toole & Kolter, 1998; Conway et al., 2002). The strains were grown overnight at 30°C both in B4M and B4Mmod media (precultures). 10 mL of the overnight cultures were transferred to 100 mL Erlenmeyer flasks containing 50 mL of the same media, and then incubated for 14 days at 30°C. The cultures were then diluted with the same liquid media to a working concentration of  $10^6$  CFU mL<sup>-1</sup>.

200  $\mu\text{L}$  of each culture was aliquoted per well on a 96-well polystyrene microtiter plate (Falcon; Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Microtiter plates with fitted lids were incubated at 30°C for 24, 48, and 72 h in a closed humidified plastic container. Uninoculated B4M or B4Mmod media were used as a blank.

The plates were then washed with saline solution (NaCl 9 g/L) to remove planktonic bacteria; the remaining biofilm-associated cells were stained for 20 min with 125  $\mu\text{L}$  1% (w/v) crystal violet. After thorough washing with saline solution, 200  $\mu\text{L}$  of 98% (v/v) ethanol was added to the well at room temperature for 15 min to release the dye in solution. The extent of biofilm, related to the crystal violet bound to biofilm-associated cells, was determined by measuring the absorbance of the resulting solution at 590 nm. For each experiment, background staining was corrected by subtracting the value for crystal violet bound to blanks. All experiments were done in at least eight replications. The data presented are the means of eight experiments.

### Statistical Analysis

A Bonferroni *t*-test was carried out with the Origin Pro 7.5 program to obtain *p*-values in assessing the significance for the differences between means of biofilm formation. Comparisons were made between each strain and the different incubation time (24, 48, 72 h). *P*-values  $\leq 0.05$  were designed to indicate a significant effect on biofilm formation, with values  $\leq 0.01$  being very significant and values  $\leq 0.001$  being extremely significant. All *p* values  $\geq 0.05$  were designed insignificant.

### CPS and EPS Extraction and Biochemical Analysis

Extracellular polymeric materials (CPS and EPS) were extracted as described by Ercole et al. (2007), with some modifications to improve the protein extraction yield from extracellular polymeric materials.

Individual colonies from slant tubes were transferred to B4M or B4Mmod liquid media and then incubated for 24 h at 30°C (starter cultures). Cultures were inoculated (10 mL) in 250 mL Erlenmeyer flasks containing 90 mL of B4M or B4Mmod liquid media and incubated for 14 days at 30°C.

After incubation, bacterial cultures were centrifuged at  $30 \times g$  for 4 min to remove mineral precipitate. The supernatant was then centrifuged at  $2,300 \times g$ , at  $4^\circ\text{C}$ , for 30 min. The resulting supernatant (i) and pellet (ii) were processed separately.

The supernatant (i), containing EPS, protein, glycoprotein, and microbial cell residues, was mixed with phosphate-buffered saline (NaCl 8 g/L, KCl 0.2 g/L,  $\text{Na}_2\text{HPO}_4$  1.44 g/L,  $\text{KH}_2\text{PO}_4$  0.24 g/L, one-fifth of the initial volume, Panreac Quimica, Barcelona, Spain) containing protease inhibitors (Ercole et al., 1999) and incubated at  $4^\circ\text{C}$  for 4 days. The mixture was then centrifuged at  $9,200 \times g$  at  $4^\circ\text{C}$  for 20 min. Microbial cells present in the pellet were discarded. The EPS fraction was isolated from the supernatant by incubating overnight with three volumes of ethanol at  $-20^\circ\text{C}$  and then centrifuging the solution at  $13,200 \times g$  at  $4^\circ\text{C}$  for 30 min. The pellet obtained, containing ethanol-insoluble EPS, was resuspended in PBS, using 1/10 of the initial volume, with the addition of protease inhibitors. The solution was treated with thioglycolic acid (0.1 M) at pH 8 and room temperature for 30 min to cleave disulfide bridges in the proteins and glycoproteins present in the mucous secretion. The solution was dialyzed for 48 h against tap water and then 72 h against distilled water, frozen at  $-80^\circ\text{C}$  and then utilized for further experiments.

The pellet (ii) obtained from the first centrifugation, containing cells, CPS protein, and glycoprotein, was resuspended in Hepes-Triton X100 (10% v/v) using 1/10 of the initial volume, and incubated at  $4^\circ\text{C}$  for 3 days. The mixture was then centrifuged at  $2,300 \times g$  for 20 min at  $4^\circ\text{C}$ . The CPS containing supernatant was added with protease inhibitors and pH neutralized by Tris base (1 M, pH 11). The solution was added with ammonium sulfate (561 g/L), mixed for 24 h at  $4^\circ\text{C}$ , and centrifuged at  $13,000 \times g$  at  $4^\circ\text{C}$  for 30 min. The obtained pellet was resuspended in Tris-HCl buffer (0.1 M, pH 7.5) supplemented with protease inhibitors, and the solution was dialyzed for 24 h against Tris-saline buffer (Tris-HCl 20 mM, pH 7.5, NaCl 20 mM). After 24 h the solution was added with protease inhibitors, frozen at  $-80^\circ\text{C}$ , and then utilized for further experiments.

The amount of protein was determined by the Lowry method (Lowry et al., 1951) by using bovine serum albumin as the standard. Total carbohydrate content of the CPS and EPS fractions was determined by the anthrone reaction (Dische, 1962), using glucose as the standard.

### SDS-PAGE Analysis

Electrophoresis was performed on 10% SDS polyacrylamide gels, as described by Laemmli (1970). Samples containing total proteins present in CPS or EPS were boiled for 5 min and centrifuged at  $20,000 \times g$  for 3 min. Supernatants, each containing 10–25  $\mu\text{g}$  of proteins, were loaded on the gel and run at 200 V (Mini-Protean apparatus, Bio-Rad, Hercules, CA, USA). The gels were then stained with Coomassie Brilliant Blue R or silver (Silver Stain Kit, Bio-Rad) to visualize the proteins.

### *In Vitro* $\text{CaCO}_3$ Precipitation by Microorganisms

The calcifying bacterial strains were spread on the surface of B4M and B4Mmod solid media and incubated at  $30^\circ\text{C}$ . All experiments were carried out in triplicate with uninoculated plates as control. Plates were examined periodically for up to 14 days by optical microscopy for the presence of crystals within or around the colonies (Bouquet et al., 1973). Morphology and size characteristics of both crystals and microorganisms were analyzed by scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDS).

Alternatively, bacterial strains were cultured in liquid media (B4M and B4Mmod). Individual colonies from slant tubes were transferred to both liquid media and then incubated for 24 h at  $30^\circ\text{C}$  (starter cultures). Starter cultures were then inoculated (10 mL) in 250 mL Erlenmeyer flasks containing 90 mL of B4M or B4Mmod liquid media and incubated for 14 days at  $30^\circ\text{C}$ . All experiments were carried out in triplicate with uninoculated flasks as controls. The appearance of crystals was monitored macroscopically. At the end, flasks were broken and glass pieces containing crystals and microorganisms were studied by SEM and EDS.

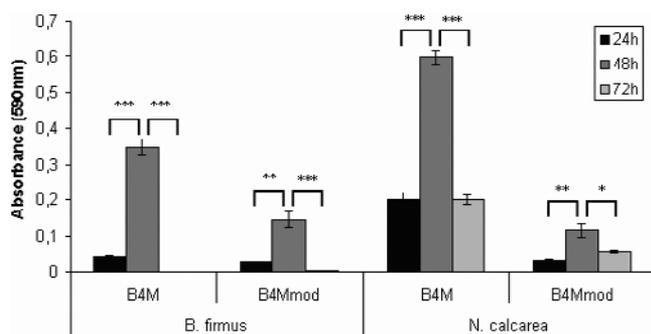
### *In Vitro* $\text{CaCO}_3$ Precipitation by Proteins Present in Extracellular Polymeric Materials

Proteins present in the CPS fraction, extracted from *N. calcarea* grown on B4M or B4Mmod liquid media, were concentrated by passage through vertical membrane concentrators [Sartorius Vivascience (Sigma-Aldrich, St. Louis, MO, USA), cut off 10,000 Da]. Aliquots of CPS fraction proteins (ranging from 0.76 to 88  $\mu\text{g}$ ) were transferred onto pieces of nitrocellulose membrane (porosity 0.45  $\mu\text{m}$ ). The nitrocellulose membranes were placed in individual petri dishes for 4 h to absorb the protein fractions and then wetted with 4 mL of sterile calcifying solution A (Ercole et al., 2007) to allow  $\text{CaCO}_3$  crystal formation at room temperature and humidity. All experiments were carried out in triplicate. Controls consisted of (1) calcifying solution A alone, not inoculated with the bacterial fractions, and (2) CPS or EPS fractions resuspended and incubated in PBS (absence of calcifying solution A). All experiments were conducted at room temperature until visible crystals were obtained. The crystals formed on the nitrocellulose membrane were examined by SEM and analyzed with an EDS.

### SEM and EDS Analysis

Morphology and size characteristics of crystals deposited in the presence of both living bacterial cells, grown on B4M solid and liquid media, and in the presence of extracellular polymeric fractions, were studied by SEM (Philips XL30CP; Philips, Guildford, Surrey, UK). The elementary composition of crystals was determined qualitatively by EDS (Oxford Link An10000) to exclude any artifacts due to the presence of particles containing elements other than carbon, oxygen, and calcium (e.g., airborne dust incorporated during SEM sample preparation).

SEM and EDS samples were prepared from: (1) agarized medium (B4M and B4Mmod), containing crystals de-



**Figure 1.** Average data for the conventional method of determining the change in biofilm formation by *B. firmus* and *N. calcearia* strains between 24, 48, and 72 h. Cells were cultured at 30°C on B4M and B4Mmod media to allow for biofilm formation. Uptake of dye was measured by a spectrophotometer, at a wavelength of 590 nm. The data illustrated are the means and standard errors of the optical densities that are correlated to biofilm formation. The significance of biofilm formation differences in each strain between treatments and different time point, 24, 48, and 72 h, was tested using the Bonferroni *t*-test. The obtained *p*-values were utilized to evaluate the statistical significance indicated in the graph by asterisks above the bars: \* represents a statistically significant effect on biofilm formation, when  $p \leq 0.05$ , \*\* represents a very significant difference, when  $p \leq 0.01$  and \*\*\* represents an extremely significant difference when value are  $p \leq 0.001$ . Values of  $p \geq 0.05$  were considered insignificant and were not marked with asterisks.

posited within and around the colonies, cut into 1 × 1 cm blocks, (2) flask pieces (1 × 2 cm) containing crystals deposited by bacterial strains (see the *In Vitro* CaCO<sub>3</sub> Precipitation by Microorganisms section), (3) nitrocellulose pieces (1 × 1 cm) containing visible crystals deposited by the CPS fraction isolated from *N. calcearia* (see the *In Vitro* CaCO<sub>3</sub> Precipitation by Proteins Present in Extracellular Polymeric Materials section).

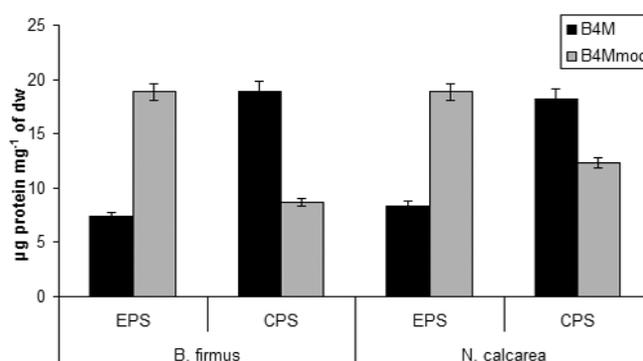
Samples and relative blanks were dried at 37°C for 10–30 days, fixed on adhesive tape sputtered with gold and observed with SEM or analyzed with EDS.

## RESULTS

### Biofilm Formation

Colorimetric assays based on crystal violet staining of biofilm were used to check for and to quantify biofilm formation and, particularly, to investigate the influence of calcium ions on biofilm production. These studies revealed that the presence of calcium ions in B4M medium increased biofilm production in both bacteria strains (Fig. 1).

Biofilm production by *B. firmus* and *N. calcearia* was, respectively, about two and six times larger when bacteria were grown on a medium containing calcium ions than when grown in the absence of calcium. Both bacteria showed a statistically significant increase in biofilm formation within the first two days, with the highest level after 48 h. However, when incubation time was extended to 72 h, strains showed a significant decrease in biofilm concentration. The reason



**Figure 2.** Protein contents versus cellular dry weight (dw) of the CPS and EPS fractions extracted from *B. firmus* and *N. calcearia* strains grown on B4M and B4Mmod media, after 14 days.

underlying this decrease remains unclear. A possible explanation is that, at this late phase, the biofilm formed started to shed pieces of extracellular material, as documented by studies of Boyd and Chakrabarty (1995). This observation can also be ascribed to a decrease in the amount of biofilm due to the lack of nutrient or space availability, as reported by Thimodo (2007).

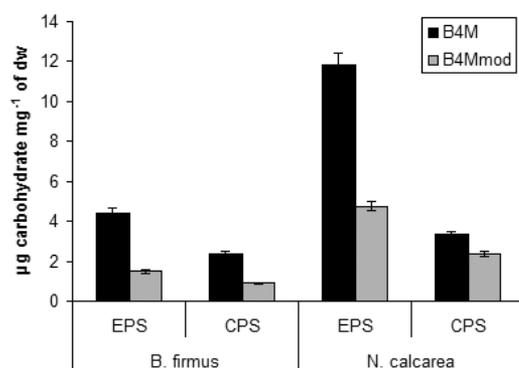
### Separation of CPS and EPS

The separation of both CPS and EPS components from *B. firmus* and *N. calcearia* was achieved by using different physical and chemical methods, as described above. Quantitative data analysis for CPS and EPS fractions is shown in Table 1. The amounts of CPS and EPS fractions extracted from bacterial cells grown on B4M medium were greater with respect to those extracted from strains grown on B4Mmod medium, lacking calcium ions.

The CPS and EPS fractions extracted from bacteria grown for 14 days on medium with or without calcium ions were assayed for protein content (Fig. 2). As reported previously (Ercole et al., 2007) in Bacilli, the maximum yield for carbohydrate and protein content in CPS and EPS fractions was obtained after 14 days of growth. The protein content of the CPS fraction isolated from *B. firmus* grown on calcium ion-containing medium was about three times larger than the CPS fraction isolated from bacteria grown in the

**Table 1.** Weight of CPS and EPS Fractions of *B. firmus* and *N. calcearia* Grown on B4M and B4Mmod after 14 days.

Bacterial Strains	EPS and CPS Fractions	Weight of EPS and CPS Fractions ( $\mu\text{g mL}^{-1}$ of Culture)
<i>B. firmus</i>	CPS B4M	13,150 ± 0.1
	CPS B4Mmod	12,700 ± 0.1
	EPS B4M	8,000 ± 0.1
	EPS B4Mmod	7,400 ± 0.1
<i>N. calcearia</i>	CPS B4M	14,300 ± 0.1
	CPS B4Mmod	12,900 ± 0.1
	EPS B4M	7,000 ± 0.1
	EPS B4Mmod	5,500 ± 0.1



**Figure 3.** Carbohydrate contents versus cellular dry weight (dw) of the CPS and EPS fractions extracted from *B. firmus* and *N. calcarea* strains grown on B4M and B4Mmod media, after 14 days.

absence of calcium ions. In *N. calcarea*, the protein content in the CPS fraction was about two times greater in the presence of calcium ions than in their absence (Fig. 2). The EPS fractions of both strains showed a differing behavior because protein amount was two times higher when cells were grown in absence of calcium ions (Fig. 2).

Carbohydrate content in extracellular polymeric fractions isolated from *B. firmus* showed a similar trend to fractions isolated from *N. calcarea* (Fig. 3). A greater level of carbohydrate expression was observed in the CPS and EPS fractions of both bacteria, grown in the medium containing calcium.

### SDS-PAGE Analysis

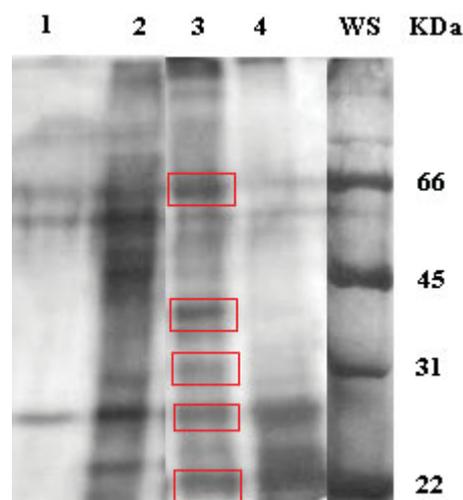
The outer-cell organic matrix was extracted from bacterial strains grown on B4M and B4Mmod media for 14 days. SDS-PAGE profiles of the proteins present in the CPS and EPS fractions extracted from bacteria grown on both media are reported in Figures 4 and 5.

Although SDS-PAGE showed that CPS and EPS fractions of both bacteria shared several common bands, each fraction was characterized by its own specific protein profile. SDS-PAGE analysis of the CPS fraction of *B. firmus* grown in the presence of calcium ions revealed that few proteins, ranging from 20 to 30 kDa and from 40 to 66 kDa, were overexpressed compared to the same fraction obtained from bacteria grown in absence of calcium ions (Fig. 4, lines 3 and 4).

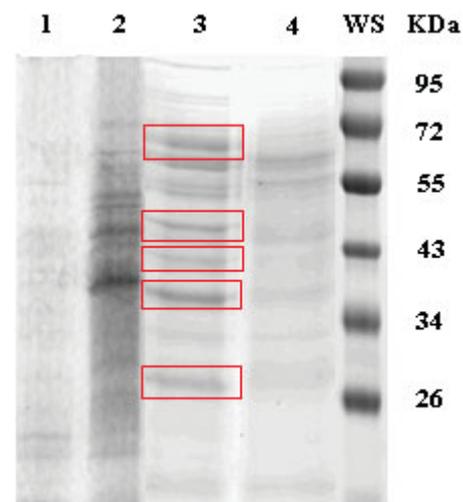
Similarly, SDS-PAGE analysis of the CPS fraction of *N. calcarea* grown in the presence of calcium ions revealed that some proteins ranging from 25 to 70 kDa were overexpressed (Fig. 5, lines 3 and 4).

On the contrary, SDS-PAGE analysis of the EPS fractions obtained from *B. firmus* and *N. calcarea* showed a general underexpression of proteins when bacteria were grown on B4M medium compared to bacteria grown in absence of calcium ions (Figs. 4 and 5, lines 1 and 2).

Overall, the presence of calcium ions in the growing medium results in a general underexpression of EPS proteins, confirming the reduction in the protein amount in this fraction above reported, and a selective overexpression



**Figure 4.** SDS-PAGE protein profiles obtained from the CPS and EPS fractions extracted from *B. firmus* strain grown on B4M or B4Mmod media for 14 days. Lines 1 and 3: proteins present in the EPS and CPS fractions isolated from bacterial cells grown on B4M medium. Lines 2 and 4: proteins present in the EPS and CPS fractions isolated from bacterial cells grown on B4Mmod medium. WS: weight standards.

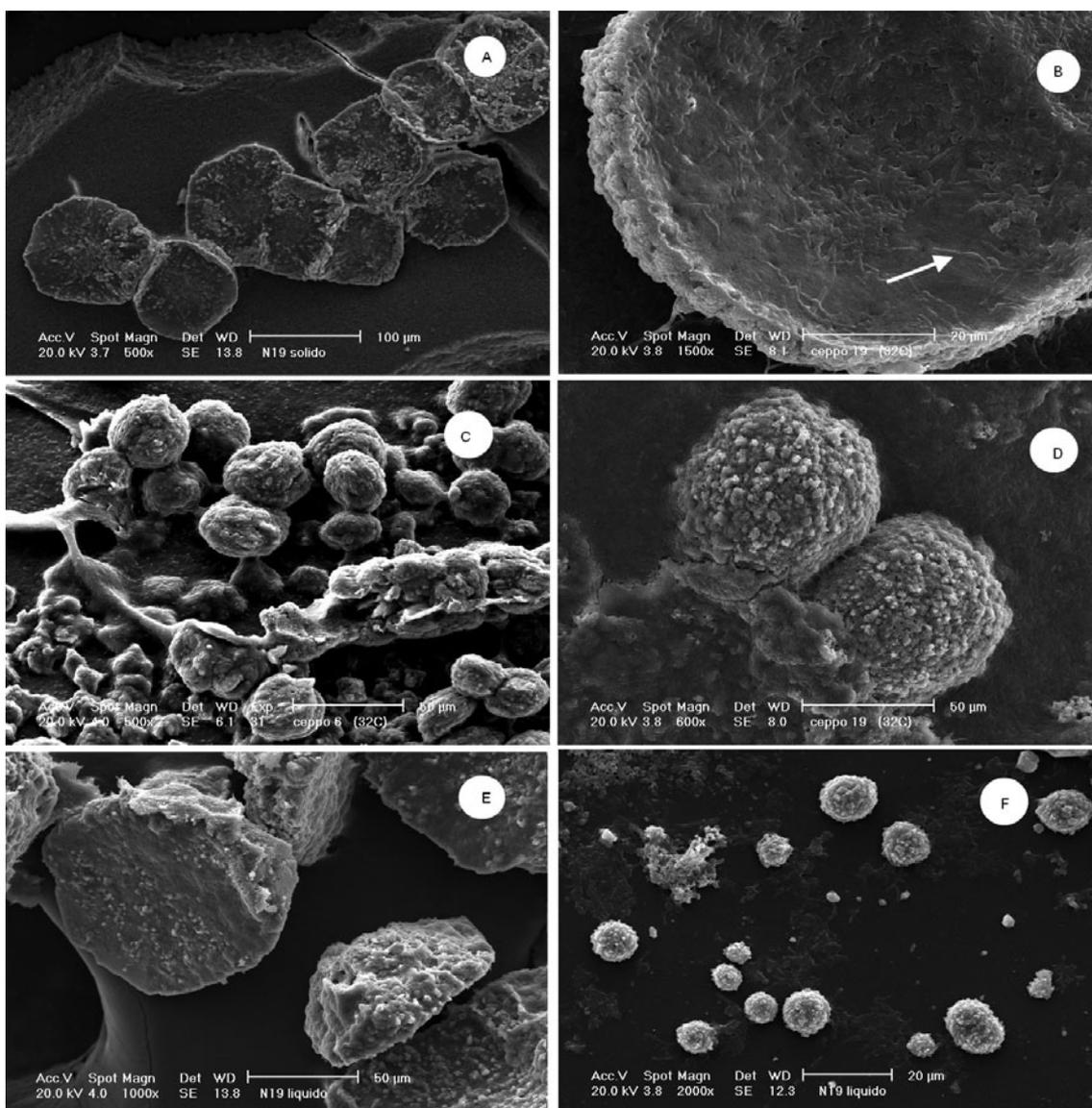


**Figure 5.** SDS-PAGE protein profiles obtained from the CPS and EPS fractions extracted from *N. calcarea* strain grown on B4M or B4Mmod media for 14 days. Lines 1 and 3: proteins present in the EPS and CPS fractions isolated from bacterial cells grown on B4M medium. Lines 2 and 4: proteins present in the EPS and CPS fractions isolated from bacterial cells grown on B4Mmod medium. WS: weight standards.

of CPS protein in both *B. firmus* and *N. calcarea* strains. These data suggest that calcium ions modulate the protein composition of the extracellular fraction, and this could be related to the active process of bioprecipitation.

### Crystal Precipitation

We checked the calcifying ability on solid and liquid culture of both bacteria. *N. calcarea* and *B. firmus* living bacterial cells formed crystals on solid and liquid B4M medium



**Figure 6.** Scanning electron micrographs of calcite crystals precipitated by living cells of *N. calcaea* grown on (A, B, C, D) B4M solid medium or on (E, F) B4M liquid medium.

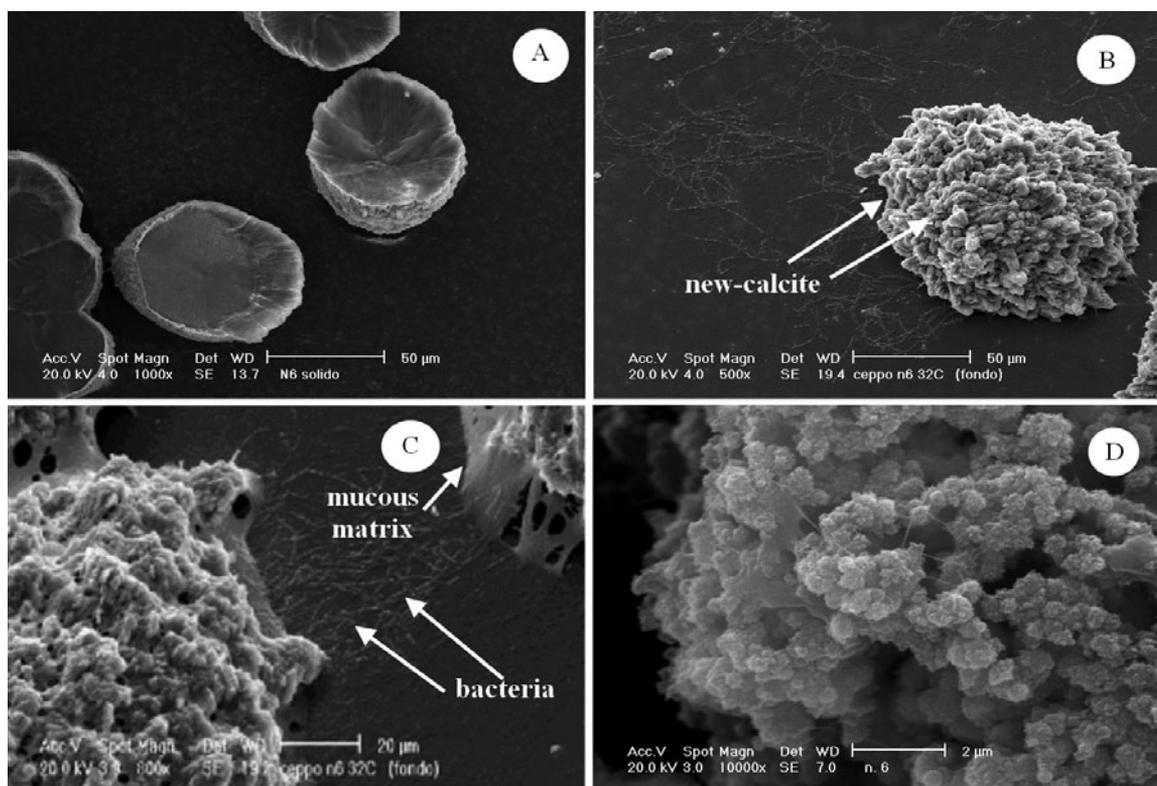
within 30 hours and 14 days, respectively (Figs. 6, 7). These results are in agreement with those obtained by Rivadeneyra et al. (2004), who investigated the precipitation of carbonates by *Halobacillus trueperi* in both liquid and solid media at different salt concentrations and found that physical state of the medium can influence the time required to initiate precipitation. Solid media or those with the highest viscosity could enable the bacteria to capture  $\text{Ca}^{2+}$  ions more selectively and to create a microenvironment more suitable for precipitation. Thus, the bacterial strains will require shorter time for bioprecipitation in solid than in liquid medium.

Scanning electron micrographs showed that living bacterial cells (Figs. 6, 7) and their CPS fractions (Fig. 8) induced the precipitation of calcite.

In Figure 6 crystals formed by *N. calcaea* grown on B4M solid medium (Figs. 6A, 6B, 6C, 6D) or B4M liquid

medium (Figs. 6E, 6F) are shown. Figure 6A shows a general view of hemispherical crystals, and Figure 6B is the corresponding magnification of a single crystal. It should be noted that crystals are mostly covered with bacteria. Figure 6C shows a general view of spherical crystals, and Figure 6D is the corresponding magnification. In this last picture the roughness present on the crystal surfaces is due to the deposition of new calcite. When the bacterial strain was incubated on B4M liquid medium, SEM revealed the same crystal shapes of solid medium, hemispherical, and spherical (Figs. 6E, 6F).

Figure 7 shows crystals formed by *B. firmus* grown on B4M solid medium (Fig. 7A) or B4M liquid medium (Figs. 7B, 7C). As observed for *N. calcaea*, crystals were either spherical (Fig. 7B) or hemispherical (Fig. 7A). Figure 7B shows that the biolith surface was covered with a layer of microcrystals of new calcite. The arrows on Fig-



**Figure 7.** Scanning electron micrographs of calcite crystals precipitated by living cells of *B. firmus* grown on (A) B4M solid medium or on (B,C) B4M liquid medium. In panel C, arrows indicate biofilm and some bacterial cells. In panel D, crystal precipitated by EPS fraction extracted by *B. firmus*.

ure 7C indicate the mucous matrix deposited between the crystals and the glass surface of the flask, as well as bacterial cells around the crystals. SEM observations of the mineral phase produced by cells, showed an intimate association between the mucous matrix induced by living cells and the mineral. It is interesting that both bacteria initiated precipitation more rapidly in solid medium (B4M) than in the corresponding liquid medium (B4M). However, when bacterial strains were incubated on solid or liquid B4Mmod media, mineral depositions were not observed.

We here show that deposition of calcite crystals was also promoted by isolated extracellular polymeric materials.

EPS extracted from *B. firmus* grown on liquid medium (B4M) are able to form a crystalline mineral when incubated on calcifying solution (Fig. 7D). In this figure a group of intergrowing in bunched form bioliths appeared embedded by this matrix.

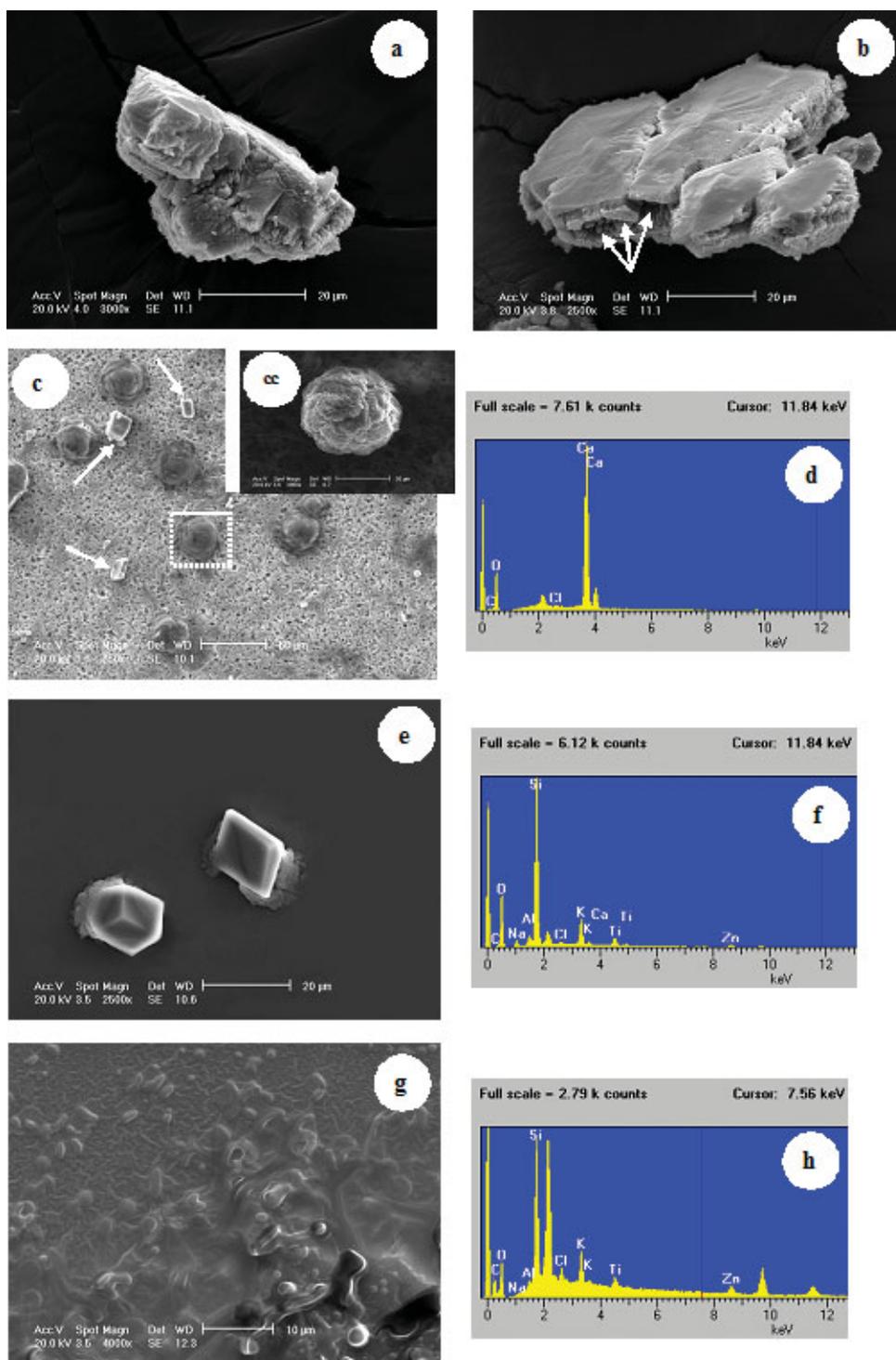
Figures 8a, 8b, and 8c show that the shape of the crystals precipitated by the CPS fractions extracted from *N. calcarrea* grown on liquid medium (B4M) and incubated in calcifying solution was very similar to that of crystals precipitated by living cells (Figs. 6E, 6F).

In Figure 8c both spherical and rhombohedral crystals can be observed. The spherical crystals are bioliths precipitated by the CPS fraction, while rhombohedral (arrows) crystals are of abiotic origin. Figure 8c (inset cc) is a magnification showing a spherical biolith.

The rhombohedral shape of crystals present in the calcifying solution A is shown in Figure 8e (blank solution), and this shape was maintained when calcifying solution was incubated with CPS fractions extracted from *N. calcarrea* (Fig. 8c). When CPS and EPS fractions extracted from both bacteria grown on liquid medium lacking calcium ions (B4M mod) were incubated in calcifying solution, no precipitate was observed. Also, no precipitate was formed when the CPS fraction extracted from *N. calcarrea* grown on B4M was resuspended and incubated in PBS buffer as a control (Fig. 8g).

EDS analysis on the crystals precipitated by the CPS fraction (Fig. 8d) revealed a chemical composition with three main elements (Ca, C, O) and a chemical formula expected for  $\text{CaCO}_3$ . These crystals were therefore characterized as calcite as confirmed by X-ray diffraction analysis (data not shown). EDS spectra were also obtained for control experiments (calcifying solution A incubated alone and CPS fraction incubated in PBS) (Figs. 8f, 8h). The results indicated that in calcifying solution A without bacteria (Fig. 8f) the primary components were C, O, and Si with Ca as a secondary component. The Si detected by EDS is presumed to be the glass utilized as support. EDS spectra of the CPS fraction incubated in PBS buffer (Fig. 8h) revealed instead C, O, and Si as primary components.

By combining SEM with EDS we were able to obtain rapid and accurate results concerning the morphology, chem-



**Figure 8.** SEM images of calcite crystals precipitated by the CPS fraction isolated from *N. calcaea* incubated in (a, b, c) calcifying solution and (d) relative EDS spectrum. In panel b, the arrows indicate deposition of layers of calcite. In panel c, spherical and rhombohedral crystals can be observed. The spherical ones are biolith precipitated by CPS fraction while rhombohedral (arrows) are crystals present on abiotic calcifying solution. Inset cc is a magnification of the rectangular area in panel c showing a spherical biolith. SEM image of rhombohedral crystals from (e) calcifying solution and (f) relative EDS spectrum. SEM image of aliquots of the CPS fraction extracted from *N. calcaea* incubated in (g) PBS and (h) the corresponding EDS spectrum.

ical composition, and microstructure of the bioliths produced by the CPS extract from *N. calcaea*.

## DISCUSSION

In a previous work, Ercole et al. (2007) isolated bacterial outer structures from Bacilli involved in calcite precipitation. In the current study we investigated the effects of calcium ions in the medium as an environmental variable to determine the role of calcium ions in inducing the over-

expression of some proteins present in biopolymers (CPS and EPS) of *B. firmus* and *N. calcaea*. Moreover, we investigated effects of calcium ions on calcite crystal formation and relative biofilm production. Our data show that EPS and CPS isolated from both bacteria grown on basic medium in the presence of calcium ions induced calcite precipitation.

The matrix of extracellular polymeric secretions has been described to influence the calcium carbonate precipitation in a positive way (Kawaguchi & Decho, 2002; Dick

et al., 2006). In natural environments, EPS appears to play a role in the coverage of surfaces by biofilms, cell adhesion, and, possibly, capturing of calcium ions, which might result in a homogeneous layer of calcium carbonate. The importance of biofilm is in colonizing stone surface and in reacting as nucleation site for extracellular calcium carbonate precipitation (Merz-Preiss & Riding, 1999; Dick et al., 2006). It is clear from a number of studies that mutants unable to synthesize the EPS are unable to form biofilms (Allison & Sutherland, 1987; Watnick & Kolter, 1999; Sutherland, 2001), although bacteria may still attach to the surface and form microcolonies to a limited extent.

In our study the amounts of EPS/CPS and biofilm extracted from both strains, grown on a medium containing calcium ions, were higher than the same fractions extracted from bacteria grown on the same medium but lacking calcium ions. The addition of high concentrations of calcium (about 4 mM) to the growth medium resulted in significant increases in the bacterial biofilm formation, as well as changes in the CPS and EPS amount and protein composition.

A comparative electrophoretic analyses of proteins present in the CPS and EPS fractions of both *B. firmus* and *N. calcaea* grown in different media revealed several common bands as well as some proteins differently expressed. Overall, the presence of calcium ions in the growing medium results in a general underexpression of EPS proteins, confirming the reduction in protein amount in this fraction, and a selective overexpression of some CPS protein.

Patrauchan et al. (2005) observed that calcium ions cause global changes in the matrix material as well as in the cellular and extracellular protein profiles of *Pseudoalteromonas* sp. Calcium ions may also play a signaling role in bacterial gene expression, particularly in components associated with biofilm growth, mainly when calcium concentration is high.

Biochemical and physiological data confirm that calcium ions are involved in a variety of bacterial cellular processes, including cell cycles and division (Yu & Margolin, 1997), competence (Trombe et al., 1994), pathogenesis (Straley et al., 1993), motility, and chemotaxis (Onek & Smith, 1992; Tisa & Adler, 1995; Norris et al., 1996; Michiels et al., 2002). As in eukaryotic cells, mechanisms of calcium signaling in bacterial cells are based on local changes of free calcium concentration in the cytoplasm (Shemarova & Neshterov, 2005).

Living bacterial cells maintained intracellular calcium concentration at a low level up to 1,000 times lower than in the extracellular medium, and as reported by Norris et al. (1996) three factors are responsible for this behavior: the low permeability of the envelop with tightly controlled influx mechanisms, the high buffering capacity, and effective export system.

Our data support the idea that extracellular calcium concentration around bacterial cells is likely related to calcium precipitation. Data reported also suggest that calcium ions can modulate the protein composition of extracellular fraction and the higher level of synthesis of specific

protein components could be related to an active process of bioprecipitation.

SEM observations reported here reveal that living cells of both bacteria utilized in this study formed calcite crystals with different sizes and shapes. Moreover, the CPS fraction extracted from *N. calcaea* promotes the precipitation of crystals, the shape of which is similar to that of crystals deposited from *N. calcaea* living cells. On the contrary, there were no precipitates in blank samples, represented by bacteria or their CPS or EPS fractions incubated in absence of calcium ions.

Based on the morphological evidence obtained and on the sequential processes of the biolith formation described in other studies (Rivadeneira et al., 1996, 1998, 2004), we can formulate two hypotheses on the process undergoing the precipitation of bioliths:

- (1) In living cells the process begins with carbonate precipitation in the bacterial envelopes, which would act as a nucleus for biolith formation. New calcified cells would be incorporated onto these structures and progressively increase the size of bioliths. The bioliths would then undergo external and internal processes of microcrystallization, finally leading via intermediate phases, to hemispherical form (Figs. 6A, 6B). The release of the cytoplasmic content or the proteins present on the cell surface may also affect the processes, contributing to the formation of spherical shape bioliths and to the recrystallization process. The completed spherical bioliths may then receive new contributions and be covered with a fine layer of microcrystals (Figs. 6C, 6D).
- (2) We hypothesized that hemispherical and spherical shapes of crystals formed by both living cells and the CPS fractions from *N. calcaea* are the results of the activity of different proteins present in the CPS fraction that could be involved in the mechanism of carbonate crystal formation and possibly could be under genetic control. These proteins may specifically bind calcium ions and promote carbonate precipitation allowing the right environmental conditions, such alkaline pH and/or a specific ion coordination, being a nucleation site.

To better understand the function of the proteins overexpressed in the CPS fraction and their involvement in the biomineralization processes, we plan to isolate and fractionate these proteins and test *in vitro* their capability to promote the process of CaCO<sub>3</sub> precipitation. Characterization of these proteins should eventually lead to the cloning of the corresponding genes, and this will make it easier to determine their functions and mechanisms regulating their expression. The information obtained in the present study constitutes an essential step in this direction.

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## REFERENCES

- ALLISON, D.G. & SUTHERLAND, I.W. (1987). Role of exopolysaccharides in adhesion of freshwater bacteria. *J Gen Microbiol* **133**, 1319–1327.
- ANDERSON, S., APPANNA, V.D., HUANG, J. & VISWANATHA, T. (1992). A novel role calcite in calcium homeostasis. *FEBS Lett* **308**, 94–96.
- BHASKAR, P.V. & BHOSLE, N.B. (2005). Microbial extracellular polymeric substances in marine biogeochemical processes. *Curr Sci* **88**, 45–53.
- BOUQUET, E., BORONAT, A. & RAMOS-CORMENZANA, A. (1973). Production of calcite (calcium carbonate) crystal by soil bacteria is a general phenomenon. *Nature* **246**, 527–529.
- BOYD, A. & CHAKRABARTY, A.M. (1995). *Pseudomonas aeruginosa* biofilms: Role of the alginate exopolysaccharide. *J Ind Microbiol* **15**, 162–168.
- BOZZELLI, P. (2011). Utilizzo di batteri calcificanti per il biorecupero dei beni culturali [Utilization of calcifying bacteria for the bioremediation of cultural heritage]. PhD Thesis. University of L'Aquila, L'Aquila-Italy.
- BRAISSANT, O., CAILLEAU, G., DUPRAZ, C. & VERRECCHIA, E.P. (2003). Bacterially induced mineralization of calcium carbonate in terrestrial environments: The role of exopolysaccharides and amino acid. *J Sediment Res* **73**(3), 485–490.
- CACCHIO, P., ERCOLE, C., CAPPUCIO, G. & LEPIDI, A. (2003). Calcium carbonate precipitation by bacterial strains isolated from a limestone cave and from a loamy soil. *Geomicrobiol J* **20**, 85–98.
- CASTANIER, S., LE METAYER-LEVREL, G. & PERTHUISOT, J.P. (1999). Ca-carbonates precipitation and limestone genesis—The microbiogeologist point of view. *Sediment Geol* **126**, 9–23.
- CASTANIER, S., LE METAYER-LEVREL, G. & PERTHUISOT, J.P. (2000). Bacterial roles in the precipitation of carbonate minerals. In *Microbial Sediments*, Riding, R. & Awramik, S.M. (Eds.), pp. 32–39. Berlin: Springer-Verlag.
- CONWAY, B.D., VENU, V. & SPEERT, D.P. (2002). Biofilm formation and acyl homoserine lactone production in the *Burkholderia cepacia* complex. *J Bacteriol* **184** (20), 5678–5685.
- DECHO, A.W. (2009). Overview of biopolymer-induced mineralization: What goes on in biofilms? *Ecol Eng* **30**, 1–8.
- DICK, J., DE WINDT, W., DE GRAEF, B., SAVEYN, H., VEN DER MEEREN, P., DE BELIE, N. & VERSTRAETE, W. (2006). Biodeposition of a calcium carbonate layer on degraded limestone by *Bacillus* species. *Biodegradation* **17**, 357–367.
- DISCHE, Z. (1962). General color reactions. *Method Carbohydr Chem* **1**, 477–479.
- DITTRICH, M. & SIBLER, S. (2010). Calcium carbonate precipitation by cyanobacterial polysaccharides. In *Tufas and Speleothems: Unravelling the Microbial and Physical Controls*, Pedley, H.M. & Rogerson, M. (Eds.), Special Publications 336, pp. 51–63. London: Geological Society.
- DOUGLAS, S. & BEVERIDGE, T.J. (1998). Mineral formation by bacteria in natural microbial communities. *FEMS Microbiol Ecol* **26**, 79–88.
- DUPRAZ, C., REID, R.P., BRAISSANT, O., DECHO, A.W., NORMAN, R.S. & VISSCHER, P.T. (2009). Processes of carbonate precipitation in modern microbial mats. *Earth Sci Rev* **96**, 141–162.
- ERCOLE, C., ALTIERI, F., PICCONE, C., DEL GALLO, M. & LEPIDI, A. (1999). Influence of manganese dioxide and manganic ions on the production of two proteins in *Arthrobacter* sp. *Geomicrobiol J* **16**, 95–103.
- ERCOLE, C., CACCHIO, P., BOTTA, A.L., CENTI, V. & LEPIDI, A. (2007). Bacterially induced mineralization of calcium carbonate: The role of exopolysaccharides and capsular polysaccharides. *Microsc Microanal* **13**, 42–50.
- ERLICH, H.L. (1996). *Geomicrobiology*, 3rd ed. New York: Marcel Dekker.
- FERRER, M.R., QUEVEDO-SARMIENTO, J., RIVADENEYRA, M.A., BEJAR, V., DELGADO, G. & RAMOS-COEMENZANA, A. (1988). Calcium carbonate precipitation by two group of moderately halophilic microorganisms at different temperatures and salt concentrations. *Curr Microbiol* **17**, 221–227.
- GONZÁLEZ-MUÑOZ, M.T., RODRIGUEZ-NAVARRO, C., MARTÍNEZ-RUIZ, F., ARIAS, J.M., MERROUN, M.L. & RODRIGUEZ-GALLEGO, M. (2010). Bacterial biomineralization: New insights from *Myxococcus*-induced mineral precipitation. In *Tufas and Speleothems: Unravelling the Microbial and Physical Controls*, Pedley, H.M. & Rogerson, M. (Eds.), Special Publications 336, pp. 31–50. London: Geological Society.
- HAMMES, F., BOON, N., DE VILLIERS, J., VERSTRAETE, W. & SICILIANO, S.D. (2003). Strain-specific ureolytic microbial calcium carbonate precipitation. *Appl Environ Microb* **69**, 4901–4909.
- HAMMES, F. & VERSTRAETE, W. (2002). Key roles of pH and calcium metabolism in microbial carbonate precipitation. In *Reviews in Environmental Science & Biotechnology*, **1**, 3–7. The Netherlands: Kluwer Academic Publishers.
- KAWAGUCHI, T. & DECHO, A.W. (2002). A laboratory investigation of cyanobacterial extracellular polymeric secretions (EPS) in influencing CaCO<sub>3</sub> polymorphism. *J Cryst Growth* **240**, 230–235.
- LAEMMLI, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- LE MÈTAYER-LEVREL, G., CASTANIER, S., ORIAL, G., LOUBIERE, J.F. & PERTHUISOT, J.P. (1999). Applications of bacterial carbonatogenesis to the protection and regeneration of limestones in buildings and historic patrimony. *Sediment Geol* **126**, 25–34.
- LIAN, B., HU, Q., CHEN, J., JI, J. & TENG, H.H. (2006). Carbonate biomineralization induced by soil bacterium *Bacillus megaterium*. *Geochim Cosmochim Acta* **70**, 5522–5535.
- LOWRY, O.H., ROSEBROUGH, M.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with Folin phenol reagent. *J Biol Chem* **193**, 265–275.
- MACILENTI, C. (2002). Indagine sui meccanismi di calcificazione promossa da batteri [Study of the mechanisms of CaCO<sub>3</sub> precipitation by bacteria isolated in different caves]. PhD Thesis. University of L'Aquila, L'Aquila-Italy.
- MCCONNAUGHEY, T.A. & WHELAN, F.F. (1997). Calcification generates protons for nutrient and bicarbonate uptake. *Earth Sci Rev* **42**, 95–117.
- MERZ-PREISS, M. & RIDING, R. (1999). Cyanobacterial tufa calcification in two freshwater streams: Ambient environment, chemical thresholds and biological processes. *Sediment Geol* **126** (1–4), 103–124.
- MICHIELS, J., XI, C., VERHAERT, J. & VANDERLEYDEN, J. (2002). The functions of Ca<sup>2+</sup> in bacteria: A role for EF-hand proteins? *Trends Microbiol* **10**(2), 87–93.
- MORITA, R.Y. (1980). Calcite precipitation by marine bacteria. *Geomicrobiol J* **2**, 63–82.
- NICHOLS, P.D. & NICHOLS, C.A.M. (2008). Microbial signature lipid profiling and exopolysaccharides: Experiences initiated with Professor David C. White and transported to Tasmania, Australia. *J Microbiol Meth* **74**, 33–46.
- NIELSEN, P.H. & JAHN, A. (1999). Extraction of EPS. In *Microbial Extracellular Polymeric Substances: Characterization, Structure,*

- and Function, Wingender, J., Neu, T.R. & Flemming, H.C. (Eds.), pp. 49–72. Berlin, Heidelberg: Springer.
- NORRIS, V., GRANT, S., FREESTONE, P., CANVIN, J., SHEIKH, F.N., TOTH, I., TRINEI, M., MODHA, K. & NORMAN, R.I. (1996). Calcium signalling in bacteria. *J Bacteriol* **178**(13), 3677–3682.
- NOVITSKY, J.A. (1981). Calcium carbonate precipitation by marine bacteria. *Geomicrobiol J* **2**, 63–82.
- ONEK, L.A. & SMITH, R.J. (1992). Calcium and calcium mediated regulation in prokaryotes. *J Gen Microbiol* **138**, 1039–1049.
- O'TOOLE, G.A. & KOLTER, R. (1998). Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: A genetic analysis. *Mol Microbiol* **28**, 449–461.
- PAPIDA, S., MURPHY, W. & MAY, E. (2000). Enhancement of physical weathering of building stones by microbial populations. *Int Biodeter Biodegr* **46**, 305–317.
- PATRAUCHAN, M.A., SARKISOVA, S., SAUER, K. & FRANKLIN, M.J. (2005). Calcium influences cellular and extracellular product formation during biofilm-associated growth of a marine *Pseudoalteromonas* sp. *Microbiology* **151**, 2885–2897.
- PERRY, T.D., MCNAMARA, C., MITCHELL, R. & HERNANDEZ-DUQUE, G. (2003). An investigation of bacterial dissolution of Maya limestone: Biodiversity and functional analysis. In *Molecular Biology and Cultural Heritage: Proceedings of the International Conference*, Sevilla, Spain, March 4–7, 2003, Saiz-Jimenez, C. (Ed.), pp. 137–140. Rotterdam, The Netherlands: A. A. Balkema Publishers.
- RIVADENEYRA, M.A., DELGADO, R., DEL MORAL, A., FERRER, M.R. & RAMOS-CORMENZANA, A. (1994). Precipitation of calcium carbonate by *Vibrio* spp. from an inland saltern. *FEMS Microbiol Ecol* **13**, 197–204.
- RIVADENEYRA, M.A., DELGADO, G., RAMOS-CORMENZANA, A. & DELGADO, R. (1998). Biomineralization of carbonates by *Halomonas eurihalina* in solid and liquid media with different salinities: Crystal formation sequence. *Res Microbiol* **149**, 277–287.
- RIVADENEYRA, M.A., PÁRRAGA, J., DELGADO, R., RAMOS-CORMENZANA, A. & DELGADO, G. (2004). Biomineralization of carbonates by *Halobacillus trueperi* in solid and liquid media with different salinities. *FEMS Microbiol Ecol* **48**, 39–46.
- RIVADENEYRA, M.A., RAMOS-CORMENZANA, A., DELGADO, G. & DELGADO, R. (1996). Process of carbonate precipitation by *Deleya halophila*. *Curr Microbiol* **32**, 308–313.
- SARRÓ, M.I., GARCIA, A.M., RIVALTA, V.M., MORENO, D. & ARROYO, I. (2006). Biodeterioration of the Lions Fountain at the Alhambra Palace, Granada, Spain. *Build Environ* **41**, 1811–1820.
- SCHULTZE-LAM, S., FORTIN, D., DAVIS, B.S., BEVERIDGE, T.J. (1996). Mineralisation of bacterial surfaces. *Chem Geol* **132**, 171–181.
- SHEMAROVA, I.V. & NESTEROV, V.P. (2005). Evolution of mechanisms of Ca<sup>2+</sup>-signaling: Role of calcium ions in signal transduction in prokaryotes. *J Evol Biochem Phys* **41**(1), 12–19.
- STRALEY, S.C., PLANO, G.V., SKRZYPEK, E., HADDIX, P.L. & FIELDS, K.A. (1993). Regulation by Ca<sup>2+</sup> in the *Yersinia* low-Ca<sup>2+</sup> response. *Mol Microbiol* **8**, 1005–1010.
- SUTHERLAND, I.W. (2001). Biofilm exopolysaccharides: A strong and sticky framework. *Microbiology* **147**, 3–9.
- THIMODO, M. (2007). A lux/gfp dual label system for studying attachment and biofilm formation of *Enterobacter sakazakii*. *Surg* **1**, 22–28.
- TISA, L.S. & ADLER, J. (1995). Cytoplasmic free-Ca<sup>2+</sup> level rises with repellents and falls with attractants in *Escherichia coli* chemotaxis. *Proc Natl Acad Sci USA* **92**, 10777–10781.
- TROMBE, M.C., RIEUX, V. & BAILLE, F. (1994). Mutation which alter the kinetics of calcium transport alter the regulation of competence in *Streptococcus pneumoniae*. *J Bacteriol* **176** (7), 1992–1996.
- WARSCHEID, T. & BRAAMS, J. (2000). Biodeterioration of stone: A review. *Int Biodeter Biodegr* **46**, 343–368.
- WATNICK, P.I. & KOLTER, R. (1999). Steps in the development of a *Vibrio cholerae* El Tor biofilm. *Mol Microbiol* **34**, 586–595.
- YU, X.C. & MARGOLIN, W. (1997). Ca<sup>2+</sup>-mediated GTP-dependent dynamic assembly of bacterial cell division protein FtsZ into asters and polymer networks *in vitro*. *Embo J* **16**, 5455–5463.
- ZAMARREÑO, D.V., INKPEN, R. & MAY, E. (2009). Carbonate crystals precipitated by freshwater bacteria and their use as a limestone consolidant. *Appl Environ Microb* **75**, 5981–5990.
- ZANARDINI, E., ABBRUSCATO, P., SCARAMELLI, L., ONELLI, E., REALINI, M., PATRIGNANI, G. & SORLINI, C. (2002). Red stains on Carrara marble: A case study of the Certosa of Pavia, Italy. In *Art Biology and Conservation: Biodeterioration of Works of Art*, Koestler, R.J., Koestler, V.H., Charola, A.E. & Nieto-Fernandez, F.E. (Eds.), pp. 226–247. New York: The Metropolitan Museum of Art Publishing.