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Bacterially Induced Mineralization of Calcium Carbonate: The Role of Exopolysaccharides and Capsular Polysaccharides

Claudia Ercole,* Paola Cacchio, Anna Lucia Botta, Valeria Centi, and Aldo Lepidi

Department of Basic and Applied Biology, University of L'Aquila, 67010 L'Aquila, Italy

Abstract: Bacterially induced carbonate mineralization has been proposed as a new method for the restoration of limestones in historic buildings and monuments. We describe here the formation of calcite crystals by extracellular polymeric substances isolated from *Bacillus firmus* and *Bacillus sphaericus*. We isolated bacterial outer structures (glycocalix and parietal polymers), such as exopolysaccharides (EPS) and capsular polysaccharides (CPS) and checked for their influence on calcite precipitation. CPS and EPS extracted from both *B. firmus* and *B. sphaericus* were able to mediate $CaCO_3$ precipitation *in vitro*. X-ray microanalysis showed that in all cases the formed crystals were calcite. Scanning electron microscopy showed that the shape of the crystals depended on the fractions utilized. These results suggest the possibility that biochemical composition of CPS or EPS influences the resulting morphology of $CaCO_3$. There were no precipitates in the blank samples. CPS and EPS comprised of proteins and glycoproteins. Positive alcian blue staining also reveals acidic polysaccharides in CPS and EPS fractions. Proteins with molecular masses of 25–40 kDa and 70 kDa in the CPS fraction were highly expressed in the presence of calcium oxalate. This high level of synthesis could be related to the binding of calcium ions and carbonate deposition.

Key words: bacteria glycocalyx, calcite precipitation, historic monument protection, calcifying bacteria, SEM

Introduction

Bacterially induced and mediated mineralization is a research subject widely studied in the past decades (Banfield & Hamers, 1997; Douglas & Beveridge, 1998; Ehrlich, 2002). Due to its numerous consequences, bacterially induced precipitation of calcium carbonate, so-called carbonatogenesis (Rodriguez-Navarro et al., 2003), has attracted much attention from both basic and applied points of view. It has implications for (1) atmospheric CO_2 fixation through carbonate sediment formation and lithification (Chafetz & Buczynski, 1992; Folk, 1993; Krumbein, 1979; Monger et al., 1991) and dolomite precipitation (Vasconcelos et al., 1995), (2) solid-phase capture of inorganic contaminants (Warren et al., 2001); (3) pathological formation of mineral concretions, such as gallstones and kidney stones in humans (Keefe, 1976); and (4) the possibility of understanding extraterrestrial biological processes such as Martian carbonate production by bacteria (McKay et al., 1996; Thomas-Keprta et al., 1998). Recent research has focused on scientific and technological implications of bacterially induced carbonate precipitation (Rodriguez-Navarro et al., 2003). One possible application is the conservation of stone artworks (Le Mètayer-Levrel et al., 1999) and the sealing of rock surrounding oil sediments (Stocks-Fischer et al., 1999) and to consolidate sun-dried earth construction (such as adobe). All these applications are based on the use of a material formed by the same bacterial metabolic pathways involved in the formation of limestone in nature.

Bacterially induced carbonate mineralization is potentially an environmentally friendly method to protect decayed ornamental carbonate stone (Le Mètayer-Levrel et al., 1999). This new conservation method reproduces what nature has been doing for eons, because many carbonate rocks have been cemented by microbe-induced calcium carbonate precipitation.

Bacteria from various natural habitats have frequently been reported to be able to precipitate calcium carbonate both in natural and in laboratory conditions (Krumbein, 1979; Chafetz & Buczynski, 1992). Mechanisms proposed, occurring mostly in marine environments, are passive or active. They include abiotic changes in seawater chemical conditions (pH, component concentrations, bicarbonate ion production) and biotical changes driven by bacteria acting as crystallization nuclei. The primary role of bacteria in the precipitation process has been ascribed to their ability to create an alkaline environment by various physiological activities (Douglas & Beveridge, 1998; Castanier et al., 1999;

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**Corresponding author*. E-mail: claudia.ercole@univaq.it

Hammes & Verstraete, 2002). Boquet et al. (1973) showed that most soil bacteria are able to precipitate crystals of calcium carbonate when tested in a medium containing calcium acetate and that calcite production by bacteria is just a function of the media composition.

Carbonate mineral formation has been correlated with both metabolic activity and the cell wall structure of a microorganism. However, the exact mechanism and function of this process within the microbial ecology of the precipitating organism remains unresolved (Hammes & Verstraete, 2002; Rivadeneyra et al., 1994).

It is known that various bacterial species can precipitate different amounts, shapes, and types of carbonate crystals from exactly the same synthetic medium (Ferrer et al., 1988; Cacchio et al., 2003, 2004). In marine environments, outer structures such as the cyanobacterial S-layer have already been recognized as the main crystalline biostructure able to act as a nucleus for calcium carbonate growth (Schultze-Lam et al., 1992). Most microbial cells in natural environments form communities inside microbial biofilms (Kawaguchi & Decho, 2002). Therefore, exopolysaccharides (EPS) and amino acids most likely play an essential role in calcium carbonate morphology and mineralogy (Rodriguez-Navarro et al., 2003). This study investigates the potential relationships between capsular polysaccharides (CPS) and EPS and calcium carbonate crystals precipitated by conventional bacterial cultures and abiotically mediated calcium carbonate synthesis. This is done using EPS or CPS fractions isolated from *Bacillus firmus* and *Bacillus sphaericus*. These calcifying bacteria were isolated in Stiffe's Cave associated with carbonate speleothems, a karst cave located near L'Aquila, Central Italy (Ercole et al., 2001). These bacterial strains produce mucoid colonies that precipitate calcite crystals coated with a thick "mucilage" (unpubl. data, Macilenti, 2002, Ph.D. thesis). We looked for cell structures of bacteria involved in calcite crystal formation.

We describe here the isolation and identification of two types of exocellular polysaccharides (PS) produced by the bacteria: CPS, intimately associated with the cell surface, and covalently bound EPS, loosely associated with the cell and released into the surrounding environment (Forni et al., 1992). We determined the contribution of both the CPS and the EPS of *B. firmus* and *B. sphaericus* to calcite precipitation. We hypothesized that outer structure isolated from *Bacilli* play a direct role in the formation of carbonate.

Materials and Methods

Bacterial Strains

The calcifying bacterial strains used in this study were isolated from Stiffe's Cave near L'Aquila (Central Italy). In previous work, the bacteria were classified as *B. firmus* and *B. sphaericus* (Cacchio et al., 2003).

Inoculum Preparation and Culture Media

The calcifying bacterial strains were maintained on slants of B4M medium: 2.5 g calcium acetate (Carlo Erba), 4.0 g yeast extract (Biolife), 10.0 g glucose (Sigma-Aldrich), and 18.0 g agar (Biolife) per liter of distilled water (Boquet et al., 1973). Individual colonies from the slant tube were transferred to B4M-liquid medium and kept for 24 h at 30°C (starter cultures). Cultures were inoculated (10 ml) in 250-ml Erlenmeyer flasks containing 90 ml of B4M-liquid medium and allowed to grow from 7 to 21 days.

Staining of Bacterial Glycocalyx

Dyes were used to visualize the glycocalyx produced by the microorganisms. Microorganism growth from day 3 to day 7 on B4M solid medium was studied under a phase-contrast microscope after staining with alcian blue—1.0% (w/v) solution in 95% ethanol for polysaccharides—adjusted to pH 2.5 for carboxylic polysaccharides and to pH 0.5 for sulphate polysaccharides (Crayton, 1982). Neutral β-glucans were visualized with the fluorochrome calcofluor (Sigma-Aldrich), used as a 0.1% (w/v) solution in 0.5 N saline (pH 6) to assure minimal distortion of cells. The stained preparations were examined using a long-wavelength UV lamp, with a UV excitation filter (G365) and 425-nm barrier filter. A positive reaction was indicated by the emission of blue-green fluorescence. The presence of glycocalyx cells was also determined by exclusion of India ink (Duguid, 1951).

CPS and EPS Extraction and Biochemical Analysis

Polysaccharides were extracted as described by Del Gallo and Haegi (1990) with some modifications. Following growth for 14 days, bacterial cultures were centrifuged at $30 \times g$ for 3 min to remove mineral precipitate. The cultures obtained were centrifuged at $2300 \times g$ at 4°C for 20 min. Supernatant (i) and pellet (ii) were processed as follows.

Supernatant (i), containing EPS, protein, glycoprotein, and residual microbial cells, was added to phosphate-buffered saline (NaCl 8 $g\,l^{-1}$, KCl 0.2 $g\,l^{-1}$, Na_2HPO_4 1.44 $g\,l^{-1}$, KH_2PO_4 0.24 $g\,l^{-1}$, one-fifth of the initial volume; Panreac Quimica SA) and protease inhibitors (Ercole et al., 1999) (EDTA-Na2 0.5 mM, Pefabloc 0.4 mM, chymostatin 1.2 μM, E64 2 μM, and phenylmethylsulphonyl fluoride 0.1 mM; Sigma-Aldrich) and incubated at 4°C. After 4 days, the mixture was centrifuged at $9800 \times g$ at 4°C for 20 min. The pellet containing microbial cells was discarded. The EPS fraction was isolated from the supernatant by incubating overnight in 3 volumes of ethanol at 4°C and then centrifuging the solution at $15{,}300 \times g$ at 4°C for 30 min. The resulting pellet, containing ethanol-insoluble EPS, was resuspended in 1/10 of the initial volume of PBS supplemented with protease inhibitors. This solution was treated with thioglicolic acid 0.1 M (Sigma-Aldrich) at pH 8 at room temperature for 30 min to cleave disulphide bridges

present in the mucous secretion. The solution was dialyzed against tap water (for 48 h) and distilled water (for 24 h), lyophilized, and weighed (Manca et al., 1996).

The bacterial pellet (ii) from the first centrifugation (CPS, protein, and glycoprotein), was resuspended in 1/10 of the initial volume of HEPES-Triton-X 1% (Carlo Erba) and kept for 3 days at 4°C. Thereafter, cells were removed by centrifugation (2300 × *g*, 20 min, 4°C) and the CPS containing supernatant CPS was supplemented with protease inhibitors (Ercole et al., 1999), dialyzed for 72 h, lyophilized, and weighed. Del Gallo and Haegi (1990) utilized PBS for extracting CPS from *Azospirillum spp*. We utilized HEPES-Triton-X because CPS in cells of *B. firmus* and *B. sphaericus* are more strongly associated.

The total carbohydrate content in the CPS and EPS fractions was determined by the anthrone reaction (Dische, 1962), using glucose as a standard. Protein was determined by the Lowry method (Lowry et al., 1951) by using bovine serum albumin as a standard.

Figure 1. Phase-contrast microscopy of *Bacillus firmus* cells. Capsule in the 48-h cultures were visualized by negative staining with India ink.

Biomineralization Experiments

Two different calcifying solutions were used to allow $CaCO_3$ crystal formation. *Solution A*, a supersaturated bicarbonate solution, was prepared by bubbling carbon dioxide (2 l min^{-1}) through a calcium carbonate (0.12 g ml^{-1}) solution for 5 h. The solution was left at 4°C until it cleared and was then filtered through a 0.2-μm-pore-sized filter leaving a precipitate-free solution containing bicarbonate and calcium ions. *Solution B* contained $CaCl_2$ (5 mM) and $NaHCO_3$ (5 mM) at pH 8 (Hirotoshi et al., 2004). Erlenmeyer vacuum flasks (250 ml) containing 9 ml of either solution A or B were inoculated with 2 ml of either CPS or EPS extracted from bacteria. We then added tetracycline (300 μg ml^{-1}) and ampicillin (300 μg ml^{-1}). All experiments were carried out in triplicate. Controls consisted of uninoculated solutions (A-B). Residual traces of CO_2, in flasks containing solution A, were removed by vacuum pump. The flasks were then incubated at 20°C under nonshaking conditions.

SDS PAGE Analyses

Electrophoresis was carried out on 10% SDS polyacrylamide gels, as described by Laemmli (1970). CPS or EPS samples were boiled for 5 min and centrifuged at 20,000 × *g* for 3 min. The supernatants, each containing 10–25 μg of protein, were loaded onto the gel and run at 200 V (Mini-Protean apparatus, Bio-Rad). The gels were then stained with either Coomassie Brillant Blue R or silver (Silver Stain Kit, Bio-Rad) to visualize the proteins. The gels were also stained with Periodic Acid Schiff (PAS) to visualize the glycoproteins (Beeley, 1985).

SEM Analysis

Morphology and size of crystals deposited from both living bacterial cells and CPS and EPS fractions were studied by scanning electron microscopy (SEM, Philips XL30CP). SEM samples were prepared as follows: (1) agar blocks of bacterial strains grown on B4M medium were dried at 37DC and (2) CPS or EPS fractions (0.5 ml) incubated on calcifying solution (A) were dried at 37°C on a glass coverslip. Samples were gold sputtered and viewed with SEM.

Analysis of Crystal Phases

Crystals deposited from CPS were analyzed by using a two-axis X-ray diffractometer (Seifert XRD 3000). The samples were carefully ground and then placed in a Plexiglas holder (14 mm wide × 26 mm long, 1 mm deep). A Cu tube with a Ni filter was used as an X-ray source working at 40 kV, 30 mA. The primary slit system was formed by a 3-mm slit followed by a Soller and a 2-mm slit; the secondary system had the same configuration with 0.3-mm and 0.2-mm slits. The spectra were collected in the range of 22–50° in 0.05°C step-scan mode. The mineral composition of the precipitates obtained *in vitro* was determined by X-ray diffraction as previously reported (Cacchio et al., 2003).

Results

Slime Production

The bacterial strains produced mucoid colonies on B4M medium. Slimy colonies were clearly noticeable after 72 h; however, slime accumulation did not occur until 7 days. Negative staining with India ink revealed encapsulated cells (Fig. 1). Positive staining with alcian blue showed that these cells were arranged in the form of a dense glycocalyx, containing acidic heteropolysaccharides (Fig. 2). Positive staining with alcian blue at both pH 2.5 and 0.5 for *B. firmus* showed the presence of acidic polysaccharides (car-

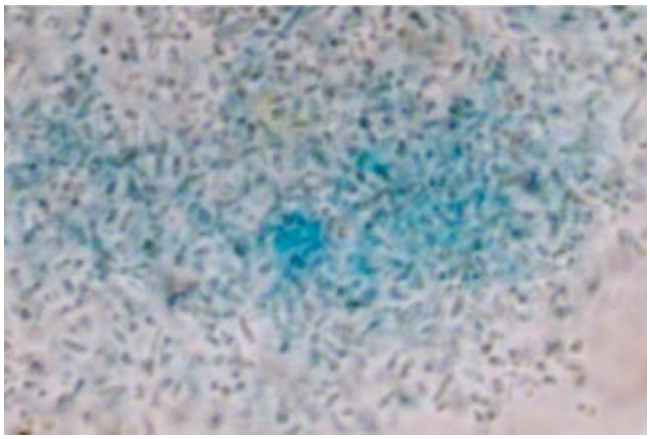

Figure 2. Phase-contrast microscopy of *Bacillus firmus* cells. Positive alcian blue staining at pH 2.5 was observed in cells when CPS and EPS were present.

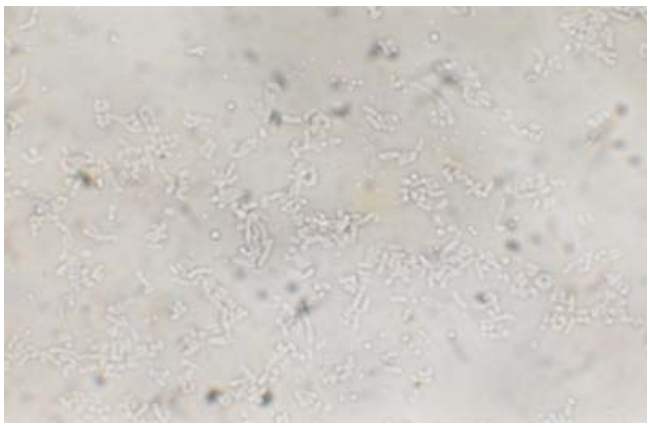

Figure 3. Phase-contrast microscopy of *Bacillus firmus* cells. Negative alcian blue staining at pH 2.5 was observed in cells after removal of the CPS and EPS fractions.

boxylic polysaccharides and sulphate polysaccharides, respectively). However, *B. sphaericus* stained with alcian blue only at pH 2.5. Removal of the CPS and EPS fractions allowed clear observation of the individual cells (Fig. 3).

Staining with calcofluor was negative, suggesting that extracellular polymeric substances contained no neutral β-glucans.

CPS and EPS Separation

Quantitative data for EPS and CPS, separated from the external surface by using differential centrifugation as described above, are shown in Table 1. The relative amounts of CPS, EPS_{sol}, and EPS_{ins} were similar in both bacteria.

Carbohydrate and protein contents in the soluble fractions (CPS and EPS) were assayed after 7, 14, and 21 days of incubation (Figs. 4 and 5). Carbohydrate content of the

Table 1. Weight of CPS and EPS Lyophilized Fractions of *Bacillus firmus* and *Bacillus sphaericus* Grown on B4M after 14 Days

Bacterial strains	EPS and CPS fractions	Weight of EPS and CPS fractions (μg ml^{-1} of culture)
Bacillus firmus	EPS_{sol}[a]	190 ± 0.01
	EPS_{ins}[b]	1390 ± 0.01
	CPS	1000 ± 0.01
Bacillus sphaericus	EPS_{sol}[a]	220 ± 0.01
	EPS_{ins}[b]	1330 ± 0.01
	CPS	980 ± 0.01

[a]EPS fraction soluble in ethanol.
[b]EPS fraction insoluble in ethanol.

different fractions showed similar trends for both *B. firmus* and *B. sphaericus*. Maximum yields of carbohydrate were obtained after 14 days of growth for both bacteria. After 21 days only the EPS_{ins} fraction in both bacteria showed an increase. CPS and EPS fractions isolated from *B. firmus* were richer in protein than those from *B. sphaericus*. The maximum amount of protein was obtained after 14 days, after which the yield remained constant (Fig. 5).

Crystal Precipitation

CPS and EPS fractions extracted from both bacteria formed crystalline $CaCO_3$ on both calcifying solutions (A and B). All crystals formed by CPS and EPS were calcite, as indicated by X-ray diffraction. Scanning electron micrographs showed that both living bacterial cells (Fig. 6A–D) and their CPS/EPS fractions (Fig. 6E–H) induced precipitates of calcite. Semispherical crystal were bound together by a mucous matrix and were induced by *B. firmus* living cells (Fig. 6A,B). Living cells of *B. sphaericus*, instead, formed crystals that were arranged as spherular or rossette-like clusters (Fig. 6C,D). EPS fractions extracted from *B. firmus* appeared to have covered the crystals as dendrite-like aggregates (Fig. 6E). Micro-krypto-crystalline aggregates of calcite with irregular borders appeared embedded in the EPS extracted from *B. sphaericus* (Fig. 6F). In CPS fractions extracted respectively from *B. firmus* and *B. sphaericus* spherular cluster of different-sized calcite crystals appeared embedded by this matrix (Fig. 6G,H). In Figure 6I, EPS fraction extracted from *B. sphaericus* and resuspended in PBS buffer (blank) is showed. It is possible to notice the absence of precipitates in the blank. The rhombohedral shape of commercially available $CaCO_3$ crystals is shown in Fig. 6J.

SDS-PAGE Analyses

Protein profiles of CPS and EPS fractions extracted from *Bacilli* grown on B4M are shown in Figures 7 and 8.

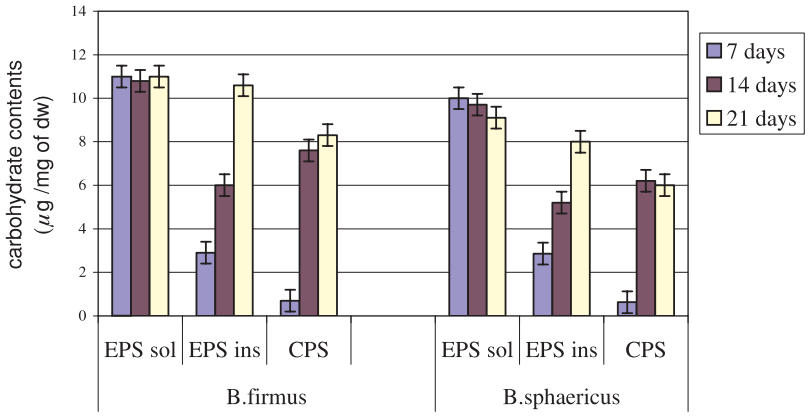

Figure 4. Carbohydrate content versus cellular dry weight for CPS, EPS_{ins} (insoluble in ethanol), and EPS_{sol} (soluble in ethanol) fractions of *Bacillus firmus* and *Bacillus sphaericus* strains grown on B4M after 7, 14, and 21 days.

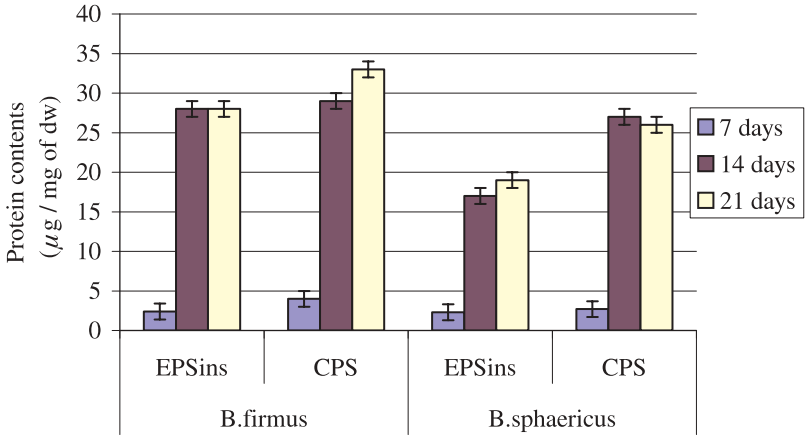

Figure 5. Protein contents versus cellular dry weight for CPS and EPS_{ins} (insoluble in ethanol) fractions extracted of *Bacillus firmus* and *Bacillus sphaericus* strains grown on B4M after 7, 14, and 21 days.

Although SDS-PAGE showed that CPS (Fig. 7) and EPS (Fig. 8) of both bacteria shared several common bands, each fraction had its own specific protein profile. When stained with Coomassie Brillant Blue SDS-PAGE, CPS fraction of *B. firmus* showed highly expressed, proteins of molecular masses of 25–40 kDa and 70 kDa (Fig. 7).

Periodic Acid Schiff staining showed (data not shown) that some proteins present in CPS and EPS fractions of both bacteria are slightly glycosylated. A band was detected for the CPS fraction of both bacteria with an apparent high molecular weight of about 77 kDa.

Discussion

The utilization of bacterial cells to preserve stone monuments has several drawbacks, such as the deposition of new products and the formation of stained patches. To overcome these problems and to improve this approach to the conservation of stone monuments, genes and proteins from calcifying bacteria should be isolated and identified in order to utilize single proteins without living cells.

The results presented here demonstrated that both *B. firmus* and *B. sphaericus* strains grown on basic medium containing calcium oxalate as the calcium source synthesize abundant extracellular polysaccharides on agar plates and liquid flocculating cultures. Amounts of CPS and EPS in both bacterial strains depends on the age of the culture, maximum yield obtained after 14 days of growth. Polysaccharides appear to be organized in two distinct morphological forms: (1) as capsules, strongly bound to the external cell surface (CPS fraction) and (2) as slimy polysaccharides, loosely attached to the capsules (EPS fraction). Even though extensive information on the role of exopolysaccharides is available for several gram-negative bacteria (Reed et al., 1988; Del Gallo et al., 1989; Whitfield, 1988), in the case of gram-positive bacteria, no information is available. It would be thus premature to define a specific role for either of the two fractions (CPS and EPS) extracted from both *Bacilli* in the process of $CaCO_3$ precipitation. Several lines of evidence indicate that microbial extracellular polymeric substances play an important role in precipitation, either through trapping and concentration of calcium ions or through the action of specific proteins that influence precipitation (Braissant et al., 2003). Kawaguchi and Decho (2002) suggested

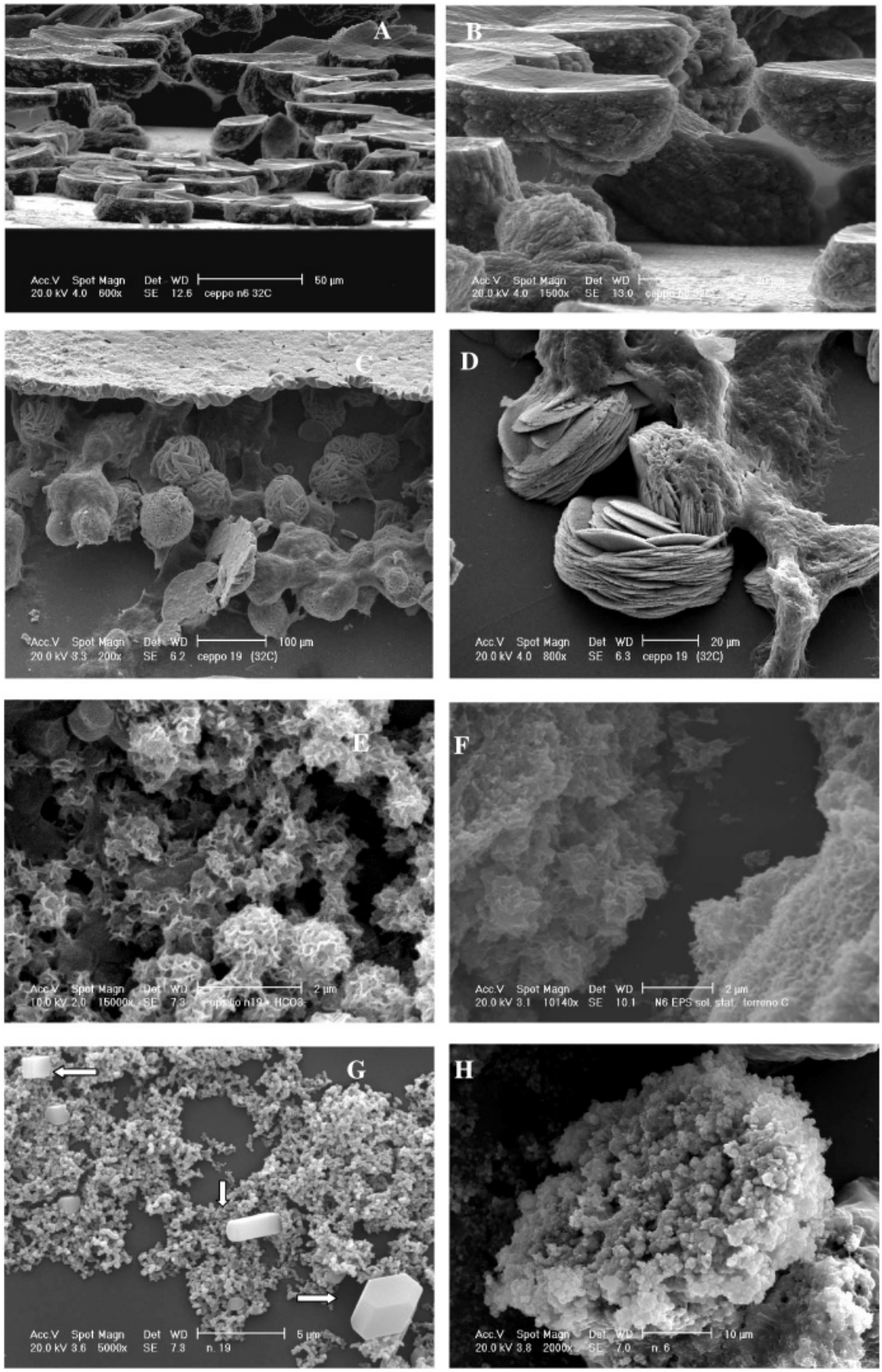

Figure 6. *Figure continues on next page.*

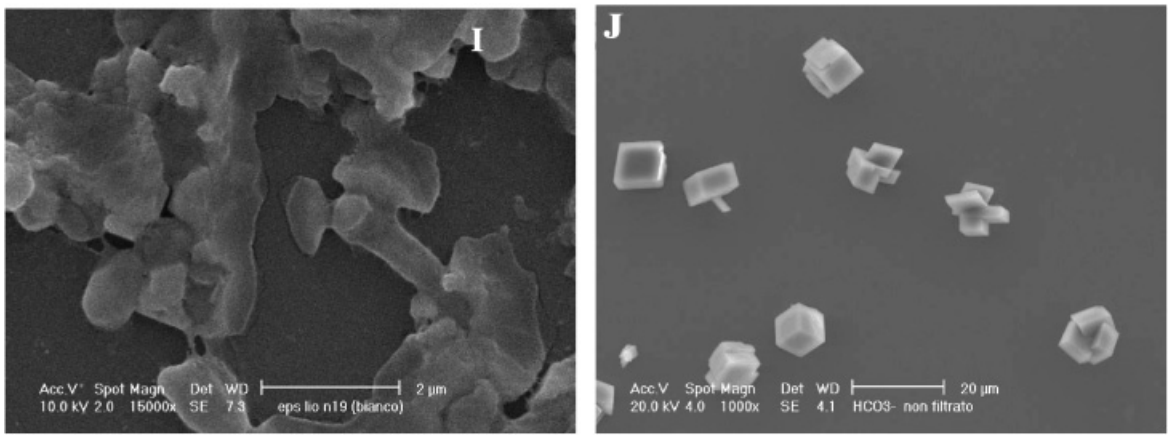

Figure 6. Scanning electron micrographs of calcite crystals precipitated by (1) living cells of *Bacillus firmus* (**A**,**B**) and *Bacillus sphaericus* (**C**,**D**) grown on B4M solid medium or by (2) CPS or EPS isolated from *Bacillus firmus* and *Bacillus sphaericus* incubated on calcifying solution (A). Calcite crystals precipitated on calcifying solution (A) by EPS fraction extracted from *Bacillus firmus* and *Bacillus sphaericus*, respectively (E,**F**). Calcite crystals precipitated on calcifying solution (A) by CPS fraction extracted from *Bacillus firmus* and *Bacillus sphaericus*, respectively (**G**,**H**). In **G**, the arrow indicates contamination of residual salt present in the buffer. EPS fraction (**I**) observed after isolation from *Bacillus sphaericus* (blank). (Note the absence of precipitates.) Rhombohedral shaped crystals of commercially available $CaCO_3$ (**J**).

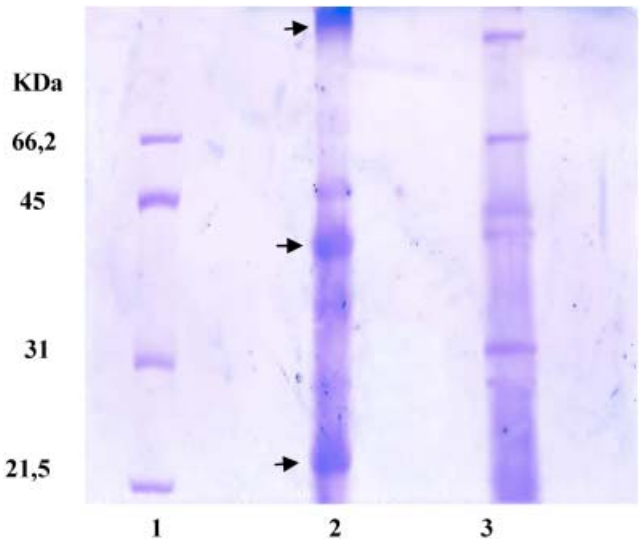

Figure 7. SDS-PAGE of soluble proteins present in CPS fractions isolated from bacterial strains grown on B4M for 14 days. Lane 1: Low range-weight standards. Lanes 2 and 3: Proteins isolated from *B. firmus and B. sphaericus*, respectively.

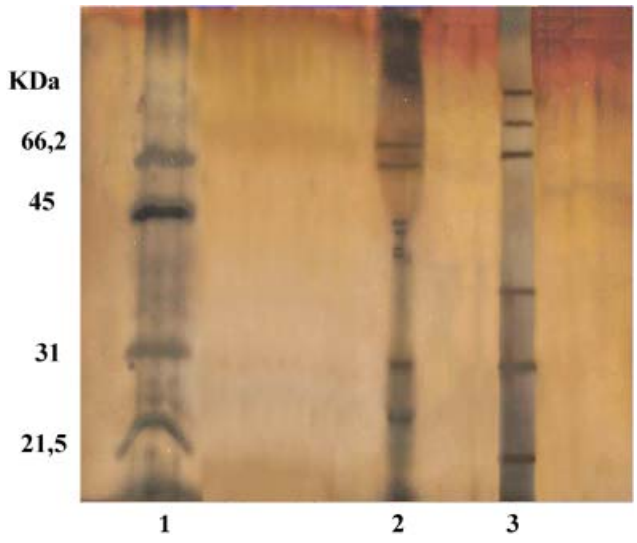

Figure 8. SDS-PAGE of soluble proteins present in EPS_{ins} fractions isolated from bacterial strains grown on B4M for 14 days. Lane 1: Low range-weight standards. Lanes 2 and 3: Proteins isolated from *B. firmus and B. sphaericus*, respectively.

that specific proteins present in biological extracellular polymeric substances cause the formation of different $CaCO_3$ polymorphs.

Although our results are preliminary, our data show that exopolysaccharides and capsular polysaccharides isolated from *B. firmus* and *B. sphaericus* play a direct role in the formation of carbonate *in vitro*. Proteins present in the CPS and EPS fractions of *B. firmus* and *B. sphaericus* revealed several common bands. Three proteins of approximate molecular mass of 25–40 and 70 kDa were synthesised in large amounts in the CPS fraction extracted from *B. firmus*. *Escherichia coli DH5* α was utilized as a control, and it showed different protein profiles with small amounts of the above quoted fractions (data not shown). Some of the proteins present in CPS and EPS from both bacteria, with an apparent molecular weight of about 77 kDa, are slightly

glycosylated and may have cation-binding potential, according the the data on glycosyled proteins reported by Albeck et al. (1996) and by Oliveira et al. (2003).

SEM images show an intimate relationship between CPS and EPS fractions and calcite crystals, as already noticed by Albeck et al. (1996; Falini et al., 1996), who report an analogous association between the shell layers of some molluscs and the crystals formed.

SEM observations showed that the shape of the newly formed crystals depends on the CPS and EPS fractions isolated from the bacteria. Also, crystals formed by living cells of both bacteria are different in size and shape from those deposited by the polymeric substances extracted from the bacterial cells. This suggests that in the mechanism of carbonate crystal formation by CPS and EPS many different fractions, such as proteins, glycoproteins, and anionic groups, may be involved in the process of bioprecipitation. Recently, in the literature, several possible interactions between crystals and organic matter are reported. Formation of magnesian calcite spherulites has been related to the slime-producing bacteria, *Myxococcus xanthus* (Gonzalez-Munoz et al., 2000). Various types of apatite have also been shown to precipitate with a spherulitic habit under the influence of organic matter or during (nano)bacterial activity (Vali et al., 2001).

The information we have obtained in this study is basis for further work. At present, we are planning experiments aimed at elucidating function and regulation of such proteins in the biomineralization processes.

The use of different synthetic media with and without calcium ions will help us to understand the role of calcium ion in the overexpression of some proteins. Isolation of these proteins enables the identification of their genes and the elucidation of a possible active role in carbonate precipitation.

Our results show new perspectives in the theoretical and practical study of biomineralization and in understanding the microbiological role in this process.

Acknowledgments

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