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Importance of B4 Medium in Determining Organomineralization Potential of Bacterial Environmental Isolates

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B4 precipitation medium has been used as the preferred medium for studying mineral precipitation using bacterial strains *in vitro* since pioneer studies were performed by Boquet and coworkers in 1973. Using this medium, several authors have demonstrated that some environmental isolates were able to precipitate minerals, yet others did not. The main goal of the current study is to understand whether pH and buffer conditions would have a significant effect on mineral precipitation results for environmental isolates grown on B4. For this study, a total of 49 strains isolated from natural environments from Puerto Rico were grown on B4 plates, and their $CaCO_3$ precipitation potential was investigated. Our findings revealed a strong correlation between a lack of $CaCO_3$ precipitation and the acidification of the B4 plates by the colonies. The ability to precipitate $CaCO_3$ could be restored by buffering the B4 medium to a pH of 8.2. Buffering capacity of the medium was proposed to be involved in $CaCO_3$ precipitation: acid-base titrations conducted on the individual ingredients of B4 showed that yeast extract has a poor buffering capacity between pH 6.5–7.5. This pH range corresponds to the pH of B4 plates [6.87 (±0.05)] prior to the inoculation. This might explain why B4 is such a good precipitation medium: a small variation in the H^+/OH^- balance during microbial growth and precipitation produces rapid changes in the pH of the medium. Finally, an amorphous matrix was distributed within 90% of the examined crystals generated on B4 medium by the environmental strains. Supplemental materials are available for this article. Go to the publisher's online edition of *Geomicrobiology Journal* to view the free supplemental file.

Keywords bacterial mineralization, B4 medium, calcium carbonate precipitation

INTRODUCTION

Mineralization, or mineral precipitation, is a common property found among many microorganisms (Dupraz et al. 2009a; Lowenstam and Weiner 1989). In uni- and multicellular eukaryotes such as bivalves (e.g., *Mercenaria mercenaria, Crassostrea gigas*), coccolithophores, and diatoms, the biomineralization is defined as *controlled* as this process is determined by intracellular organic matrices produced under specific metabolic and genetic control (Bäuerlein 2003, 2004; Bazylinski and Moskowitz 1997; Lowenstam and Weiner 1989). In contrast, *induced* organomineralization is regulated by the combined physiological activities of a microbial community carried out in open environments (Dupraz et al. 2009a; Dupraz et al. 2009b; Lowenstam and Weiner 1989; Ries et al. 2008). Metabolic pathways like photosynthesis, urea hydrolysis and sulfate reduction can result in environmental alkalinization leading to the precipitation of minerals (Braissant et al. 2007; Chafetz and Buczynski 1992; Dupraz and Visscher 2005; Hammes et al. 2003; Visscher et al. 1998; Visscher and Stolz 2005).

Several papers have addressed the ecological implications of bacterially *induced* mineral precipitation (Braissant et al. 2004; Dupraz et al. 2009b; Garvie 2006; Wright and Oren 2005). Other studies on mineral precipitation emphasize the biotechnological aspects with applications ranging from bioremediation to control of leaching (Gollapudi et al. 1995), plugging and/or

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cementation of rocks (Stocks-Fischer et al. 1999), solid-phase capture of inorganic contaminants (Warren et al. 2001), and restoration of carbonate monuments (Barabesi et al. 2003; Rodriguez-Navarro et al. 2003; Zamarreño et al. 2009a). In addition, these investigations are important for a better understanding of fossilization processes and the study of life in rocks (Benzerara and Menguy 2009).

In many organomineralization experiments with heterotrophic bacteria, the *in-vitro* generation of crystals is performed using B4 medium, which consists of 0.4% yeast extract, 1% dextrose, 0.25% calcium acetate and agar (Boquet et al. 1973). Modified versions of B4 medium have also been used; for example, calcium acetate was replaced by calcium citrate to study vaterite precipitation (Braissant and Verrecchia 2002), and by barium acetate to study barite precipitation (Sanchez-Moral et al. 2003). Dextrose levels were decreased to 0.5% to study carbonate precipitation in *Bacillus* (Barabesi et al. 2007; Marvasi et al. 2010).

B4 is the most common medium used in general organomineralization studies and has been used to assay or to characterize mineral precipitation potential. B4 medium has been used to culture microorganisms involved in active $CaCO_3$ precipitation from different ecosystems such as caves, soils, and water (Baskar et al. 2006; Braissant et al. 2002; Cacchio et al. 2004; Monger et al. 1991). B4 was also used to determine the effects of temperature on precipitation, observing that microbial $CaCO_3$ precipitation increased with time and temperature of incubation (up to 40°C) (Cacchio et al. 2003; Zamarreño et al. 2009b).

$CaCO_3$ precipitation on B4 was further affected by other factors, such as the addition of K_2HPO_4. Precipitation of $CaCO_3$ decreased when PO_4^{3-} ion concentration was increased 10-fold in culture media with complete inhibition at 1.4 g L^{-1} of K_2HPO_4 (Portillo et al. 2009; Rivadeneyra et al. 1985). B4 medium has also been utilized to assay mineralization properties of microorganisms isolated from indoor artworks and air samples (Pangallo et al. 2009). More than 50% of environmental bacterial isolates tested to date are able to precipitate $CaCO_3$ on B4, these include members of the *Bacillus*, *Arthrobacter*, *Kingella* and *Xanthomonas* (Cacchio et al. 2003, 2004; Sprocati et al. 2008; Urzì et al. 1999).

Our previous studies with a non-precipitating laboratory strain *Bacillus subtilis etfA* mutant revealed that a decrease in the pH during biofilm growth prevented $CaCO_3$ precipitation, which was always observed in wild-type *B. subtilis* 168 strain (Barabesi et al. 2007; Marvasi et al. 2010). Further analysis demonstrated that when compared to the 168 strain, the *etfA* mutant extruded an excess of 0.7 mol H^+L^{-1}. This excessive proton extrusion prevented the precipitation of $CaCO_3$ (Marvasi et al. 2010). Using media buffered at different pH values, we were able to control $CaCO_3$ precipitation by the two strains (Marvasi et al. 2010).

The goal of the current study was to investigate whether these previous observations of the impact of B4 medium made in two domesticated laboratory strains could be extended to other strains isolated from natural environments.

MATERIALS AND METHODS

Enrichment and Characterization of Wild-Type Strains

Soil samples (0.5g) from five different locations in the South of Puerto Rico were resuspended in physiological salt solution (0.8% NaCl) and inoculated onto Nutrient Agar plates (BD). The plates were incubated at 37°C for 48 h and bacterial colonies were further purified on Nutrient Agar or Luria Bertani media (Marvasi et al. 2009) in the Axenic cultures were classified according to the sampling location (A to E), followed by a number identifying the strain.

Colonies were initially classified based on morphology and chromatogenesis under a stereomicroscope (Leica ES2) and further characterized based on cell shape, and Gram type determination using standard procedures (Powers 1995) to isolate unique morphotypes. Furthermore, strains were also characterized by their capability to precipitate crystals and color variation of the B4 media (see next sections). Duplicate samples of each isolate were grown at least three times for each specific analysis.

Bacillus Strains and B4 Precipitation Media

Strains used in this study were *Bacillus subtilis* 168 (also called strain 168) (Anagnostopoulos and Spizizen 1961) and *B. subtilis* 168 mutated in *etfA* gene (strain FBC5) (Barabesi et al. 2007). *Bacillus subtilis* 168, which produces $CaCO_3$ crystals on (standard) B4 medium (Boquet et al. 1973) was used as a positive control. *B. subtilis etfA* mutant was used as negative control. Duplicate samples of each culture were grown on "standard" or on buffered B4 solid precipitation medium (0.4% yeast extract, 0.5% dextrose, 0.25% calcium acetate, 1.4% agar) according to Boquet et al. (1973). Formation of $CaCO_3$ crystals was monitored on plates incubated at 39°C inside a plastic container to prevent dehydration. The pH of liquid B4 was determined with a Hanna N2419 pH meter. *B. subtilis etfA* mutant cultures were supplemented with chloramphenicol (5 μg/ml) to maintain the selective pressure on the inserted plasmid.

pH Changes during Biofilm Development

pH changes of *B. subtilis* colonies grown on B4 plates were monitored using phenol red (PR), which exhibits a yellow color at pH 6.4 or below and a red color at pH 8.2 and above. PR stock solution (20 mg/ml in NaOH 0.1N) was added to B4 medium before autoclaving at a final concentration of 0.0025% (v/v). Experiments were replicated three times.

Precipitation Test on Buffered B4 Solid Media at Different pH

To test whether $CaCO_3$ formation was induced during biofilm growth of environmental strains at different pH, strains were

grown on modified B4 plates prepared with pH values of pH 7.3 and pH 8.2 (Marvasi et al. 2010). The pH of each plate was maintained by buffering the medium with TRIS 1.2% (w/v) and HCl 2N. PR was added to the plates to monitor pH variations during biofilm growth. Plates were incubated at 39°C and crystal formation determined after one and two weeks. Experiments were performed using three replicates for each strain.

Acid-Base Titration

First, 0.4 g of Yeast Extract (Difco) was dissolved in distilled water to obtain a final volume of 100 mL. The initial pH of the solution was adjusted to pH 5.0 and the solution was titrated with 0.1 N NaOH added stepwise in 10 or 20 μL increments. The pH was recorded at each step until pH 10 was reached using a Hanna N2419 pH meter. The titration was conducted three times.

Matrix and Crystal Fluorescence Characterization

Biofilms with crystals were collected from plates with forceps and then boiled in distilled water for 15 min to remove biofilm aggregates from the crystals. The crystals were collected by filtration and dried overnight at 37°C. The crystals were stained with crystal violet for 1 min followed by 15 sec in ethanol (70%) and washed in distilled water. Crystals were then placed on a glass with a micro cover glass and observed with a Nikon Eclipse E400 optical microscope at 100 X magnification. Mineral dissolution was tested by adding one drop of 0.1 N HCl between the glass and the cover slip. In order to detect matrix intrinsic fluorescence, images were taken using a Leica DM LB fluorescence microscope equipped with the following filters: DAPI A4 360/40, Fluorescein (L5) 480/40 and Cy3 (Y3) 535/50.

Confocal Laser Scanning Microscopy

B. subtilis 168 biofilms were grown on microscope slides carrying a thin B4 medium layer. To produce crystals on the biofilm surface, slides with B4 medium were incubated in a empty Petri plate at 38°C. Analysis of the crystals was performed with a Leica TCS SP5 confocal laser scanning microscope (Leica, Mannheim, Germany) equipped with a HeNe/Ar laser source. The fluorescent signals were obtained using 360, 480 and 535 nm excitation wavelengths in order to detect crystal fluorescence. Images of fluorescent signals were taken simultaneously and successively merged by using Leica Application Suite software. Images were further analyzed with ImageJ software.

Scanning Electron Microscopy with Energy Dispersive Spectroscopy (SEM/EDS) Analysis

To study the different crystal morphologies and elemental composition we used SEM microscopy coupled with EDS. Crystals were scraped from different biofilms and boiled for 15 min in distilled water to remove large cellular aggregates. Crystals were then collected by filtration and dried overnight at 39°C. Mineral samples were mounted on carbon tape and sputter coated with gold/palladium for 1.5 min in a Polaron Instruments SEM coating unit E5100. FEI Strata 400S dual beam FIB SEM with an EDAX Genesis was used for analysis. SEM micrographs were obtained at an accelerating voltage of 3kv (working distance 5 to 7.4 mm) and EDS was performed at 10kv.

RESULTS

Our initial isolations from environmental samples yielded approximately 100 strains. The colonies were further characterized based on morphology and pigmentation as well as Gram stain and cellular shape. Grouping based on these different parameters reduced our sample size to 49 unique morphotypes. We also used two *Bacillus subtilis* control strains (168 and FBC5). The main cell morphologies present in the environmental isolates were: 36% rods, 59% cocci and 4% diplococci. Chromogenesis of the colonies was reported as white (52%), beige (26%), yellow (20%) and orange (2%). Then, 74% of the isolated strains were Gram positive and 26% Gram negative (Supporting Information, Table S1).

To determine the $CaCO_3$ precipitating potential, each of the 49 isolates were plated in triplicate on standard B4 medium, and two buffered B4 media at pH 7.3 and 8.2, respectively. The plates were supplemented with Phenol red to indicate changes in pH during biofilm development (Fig. 1). The utilization of a pH indicator allows a fast and easy determination of whether the crystal impairment was associated with pH decreases (acidification below pH 6.4 yields a yellow color). After 2 weeks of incubation on standard B4, $CaCO_3$ crystal precipitation was observed in 61% of the strains (Table 1). An increase to 73% was obtained when the medium was buffered to 8.2. Interestingly, we were able to manipulate $CaCO_3$ formation *in vitro* as crystal formation was completely inhibited in 79% of the strains when B4 was buffered at pH = 7.3.

We identified three strains that were able to precipitate calcium carbonate on buffered B4 at pH 7.3 by the end of the second week (A17, C4, C5, media shifted to red). Alkalinization (red) of plates coincided with crystal formation, whereas acidification (yellow plates, pH 6.4 or lower) was always associated with lack of crystal formation. None of the strains developed crystals on acidic medium after a two-week incubation period. Seven strains of the environmental sample were unable to grow on B4 buffered at pH 7.3 and nine strains were unable to grow at pH 8.2. Precipitation of calcium carbonate was not correlated with a specific Gram staining or colony morphology; precipitation occurred in all phenotypes (Table S1).

SEM micrograph analysis conducted on the crystals collected from different strains grown on standard B4 revealed many differences in crystal morphologies (Fig. 2), consistent with calcium carbonate. In some crystals, calcified bacteria or their casts were well visible (A1 and B2 strains). Highly porous and globular structures were also very common (D2, E10, E15 strains).

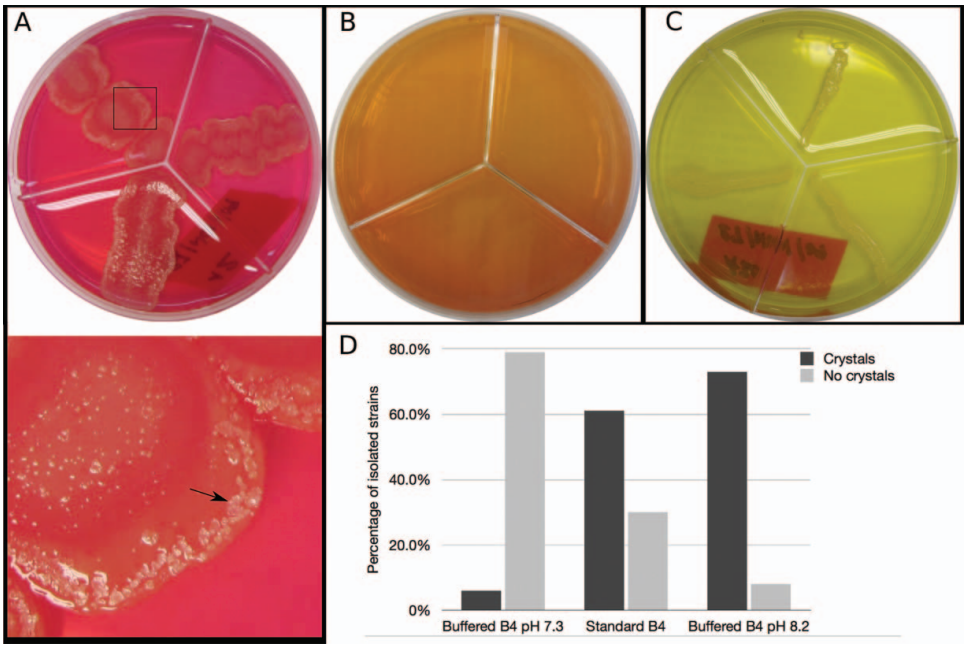

FIG. 1. $CaCO_3$ crystal formation reported on environmental strains is dependant on the buffered condition of the B4 precipitation medium. Plates were incubated for two weeks at 39°C and supplemented with the pH indicator Phenol Red. As shown in Panel A, the plates inoculated with strain A2 remained red (basic pH range) during crystal formation. Crystals are indicated by the arrow (bottom picture). Panel B is a control plate depicting the orange color developed in plates with neutral pH. Panel C shows a plate inoculated with strain A28 (the yellow coloration indicates acid pH range). Even after 2 weeks of incubation, crystals do not develop in those plates where biofilms develop under acid or neutral (orange) conditions. Panel D summarizes the percentage of environmental strains able to precipitate crystals (black bars) versus strains impaired in crystal formation (grey bars) during growth on standard B4 (not buffered, original pH 6.9), buffered B4 pH 7.3 and buffered B4 pH 8.2 media (color figure available online).

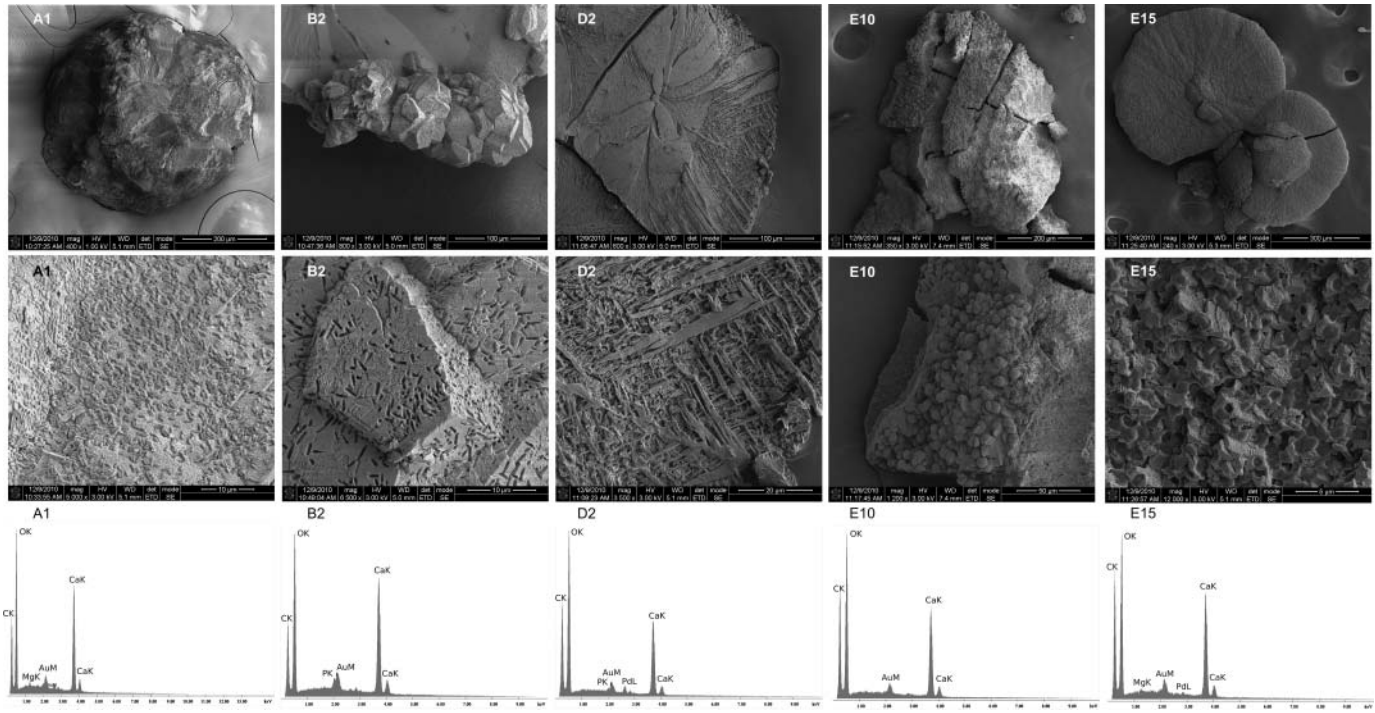

FIG. 2. SEM micrographs revealing different morphologies of crystals precipitated on biofilms from five different environmental isolates (A1, B2, D2, E10, E15) grown on standard B4. Three pictures showing an overview of the crystal, its magnification and the EDS analysis of each strain are presented. For each sample, EDS analysis showed three dominant peaks of calcium, oxygen and carbon.

TABLE 1
Crystal formation by environmental isolates and coloration of the plates after two weeks of incubation at 39°C

Strain name	Medium (14 days) Standard B4	Buffered B4 pH 7.3	Buffered B4 pH 8.2
B. Subtilis	R +	O −	R +
FBC5	O −	O −	R +
A I	R +	O −	R +
A 7	R +	O −	R +
A 12	R +	O −	R +
A 16	R +	O −	R +
A 17	R+	R +	R +
A 19	R +	O −	R +
A 24	R +	O −	R +
A 25	O −	*ng*	*ng*
A 28	Y −	O −	R +
A 29 I	R +	O −	R +
A 29 II	R +	O −	R +
B 2	R +	O −	R +
B 3 I	R +	*ng*	R +
B 3 III	*ng*	*ng*	*ng*
B 8	R +	O −	R +
B 9	R +	O −	R +
B 12	Y −	*ng*	*ng*
B 12 I	O −	*ng*	*ng*
B 17	Y −	O −	O −
B 18	Y −	O −	*ng*
B 19	R +	O −	R +
B 21	Y −	O −	O −
B 22	Y −	Y −	R +
B 23	Y −	O −	R +
B 28	Y −	O −	R +
C 1	R +	O −	R +
C 2	*ng*	O −	O −
C 3	O −	Y −	O −
C 4	R +	R +	R +
C 5	R +	R +	R +
C 8	*ng*	*ng*	*ng*
D 2	R +	O −	R +
D 3	R +	O −	R +
D 4	R +	O −	R +
E 1	R +	O −	R +
E 2	R +	O −	R +
E 4	Y −	O −	*ng*
E 6	Y −	O −	R +
E 8	R +	O −	R +
E 10	R +	O −	R +
E 15	R +	O −	*ng*
E 16	R +	O −	R +
E 20	R +	O −	R +
E 22	R +	O −	R +
E 24	R +	O −	R +
E 27 I	*ng*	*ng*	*ng*
E 27 II	Y −	O −	R +
E 29	R +	O −	R +
E 31	Y −	O −	R +

pH indicator changed to red after 1 or 2 weeks of incubation; (R) red (indicatively pH≥8.2); (Y) yellow (indicatively pH≤6.4); (O) orange; (+) crystal precipitation; (−) crystal precipitation does not occur; (*ng*) no growth. *B. subtilis* strain 168 was used as positive control and FBC5 (*etfA* mutant) as negative control.

Buffering Capacity Controls $CaCO_3$ Precipitation

The buffering capacity of the B4 medium played a critical role in supporting or preventing formation of the crystals. Therefore, we determined the buffering capacities of the medium components: due its high concentration of proteins, yeast extract was the only component able to affect the buffering capability (Nelson and Cox 2004). Titration experiments performed on yeast extract revealed two buffering ranges (Fig. 3): The first, at a range from pH 5 to 6, could be attributed to carboxyl groups and phosphate groups of the medium (Braissant et al. 2007; Stumm and Morgan 1996). The second buffering zone, between pH 8.1 and 10, is characteristic for amino groups (Phoenix et al. 2002).

Matrix Associated with the Carbonate Crystals

About 90% of the strains which precipitated $CaCO_3$ on standard B4 medium, had an amorphous, translucent, matrix associated with the carbonate crystals. Based on our light microscopic observations, this matrix could consist of extracellular polymeric substances (Marvasi et al. 2010), dead cells, and spores (of those spore-forming bacteria). A drop of 0.1 N HCl was used to completely dissolve the $CaCO_3$ and reveal the presence of an intact amorphous matrix (Fig. 4, panels C to E).

Further microscopic analysis of the matrix using CLSM revealed that the matrix was embedded in the carbonate crystals that were precipitated by *B. subtilis* 168 control strain. Organic matter incorporated in the crystals had a natural fluorescence if irradiated with the following wavelengths: 360, 480, and 535 nm (Figs. 5A–D). Calcite, a type of calcium carbonate, can be also fluorescent if excited by UV; however, in this study, the wavelengths used had much less energy than UV.

For comparison, $CaCO_3$ crystals produced through an abiotic process (forced precipitation from a $CaCl_2$ solution exposed to an ammonium carbonate-saturated headspace) were examined at these wavelengths and did not fluoresce (Figs. 5E–F). In contrast, crystals precipitated in the *B. subtilis* 168 biofilm exhibited fluorescence patterns similar to those shown in Figures 5A–D attributable to organic molecules/matter sequestered inside the crystals. CLSM analysis showed that the intrinsic fluorescence persisted internally (Figs. 5G–O). These observations suggest that the matrix can be envisioned as an internal "sponge", the holes of which are filled with $CaCO_3$.

DISCUSSION

Microbial metabolism changes the geochemical conditions of both natural and synthetic (i.e., in Petri plates) environments (Dupraz and Visscher 2005; Marvasi et al. 2010). In particular microbial $CaCO_3$ precipitation is well-documented and has been reported for a variety of bacterial species (Cacchio et al. 2003; Dupraz et al. 2009a; Nam Lee 2003; Reid et al. 2000). However, the actual geochemical conditions under which precipitation (organomineralization) occurs *in vitro* are not always clear. Historically, using B4 medium, several authors have demonstrated that some environmental isolates were

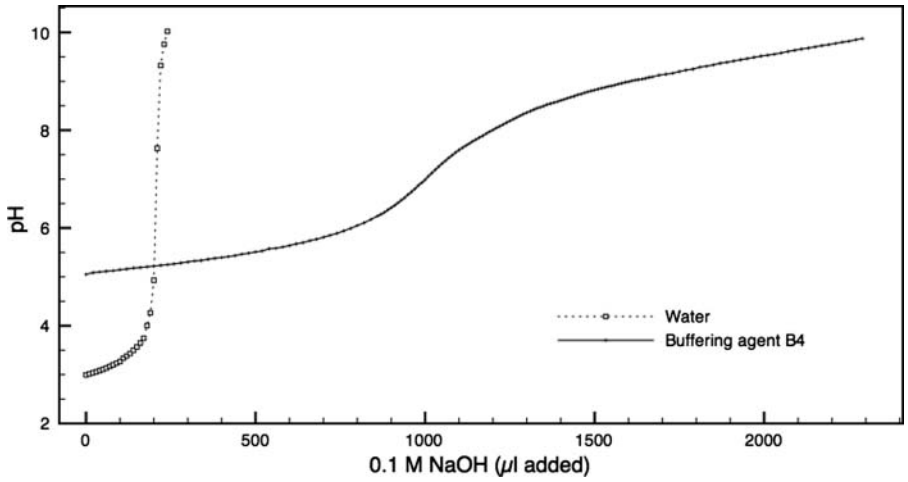

FIG. 3. Acid-base titration of yeast extract in water showing two buffering zones at pH 5–6.5 and 7.5–9 (continuous line). Control titration was conducted using an identical volume of deionized water adjusted to pH 3 with HCl (dashed line).

able to precipitate minerals while others did not (Boquet et al. 1973; Cacchio et al. 2003). Recently, Marvasi et al. (2010) showed using *B. subtilis* laboratory strain FBC5 that precipitation potential could be manipulated by changing medium pH and buffer composition. In the present study we wanted to elucidate the phenomenon of organomineralization on B4 medium, using a survey consisting of a variety of environmental isolates.

Organomineralization is defined as passive mineralization of organic matter (biogenic or abiogenic in origin), whose properties influence crystal morphology and composition (Dupraz et al. 2009a). Organomineralization is dictated by two key components, closely coupled and fundamental in the control of carbonate precipitation: 1) the so called "alkalinity engine" and 2) the presence of nucleation sites such as the extracellular organic matrix, or the surfaces of cells. (Bosak and Newman 2005; Dupraz et al. 2009a; Ferris et al. 1989).

The components of the alkalinity engine affected by microbial metabolism are pH, total alkalinity, and carbonate alkalinity. These factors, in turn, influence the Saturation Index (SI)

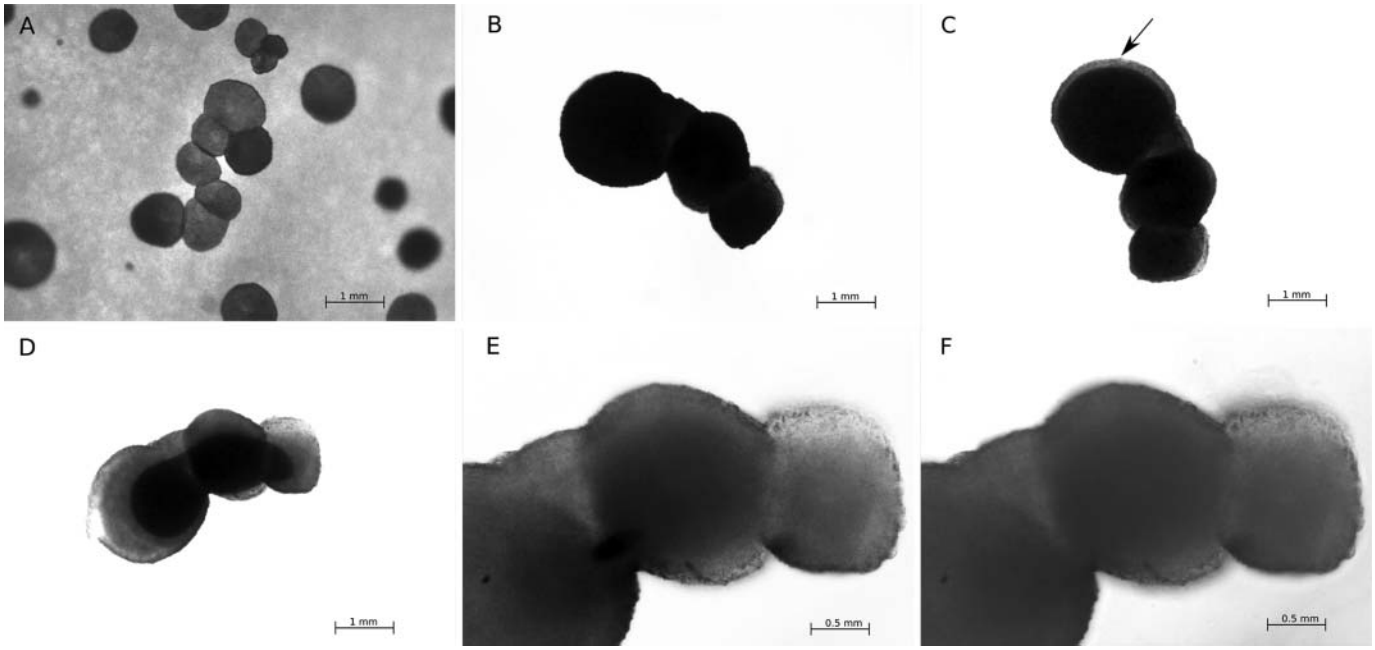

FIG. 4. Matrix associated with the carbonate crystals. Panels A and B show untreated crystals. Panels C and D show the progressive dissolution of the carbonate crystals following 0.1 N HCl addition. The arrow in Panel C indicates the onset of dissolution of the crystal (dark) leaving the amorphous matrix (opaque). Panels D and E show a time serial observation of the progressive $CaCO_3$ dissolution with complete dissolution of the $CaCO_3$ shown in Panel F.

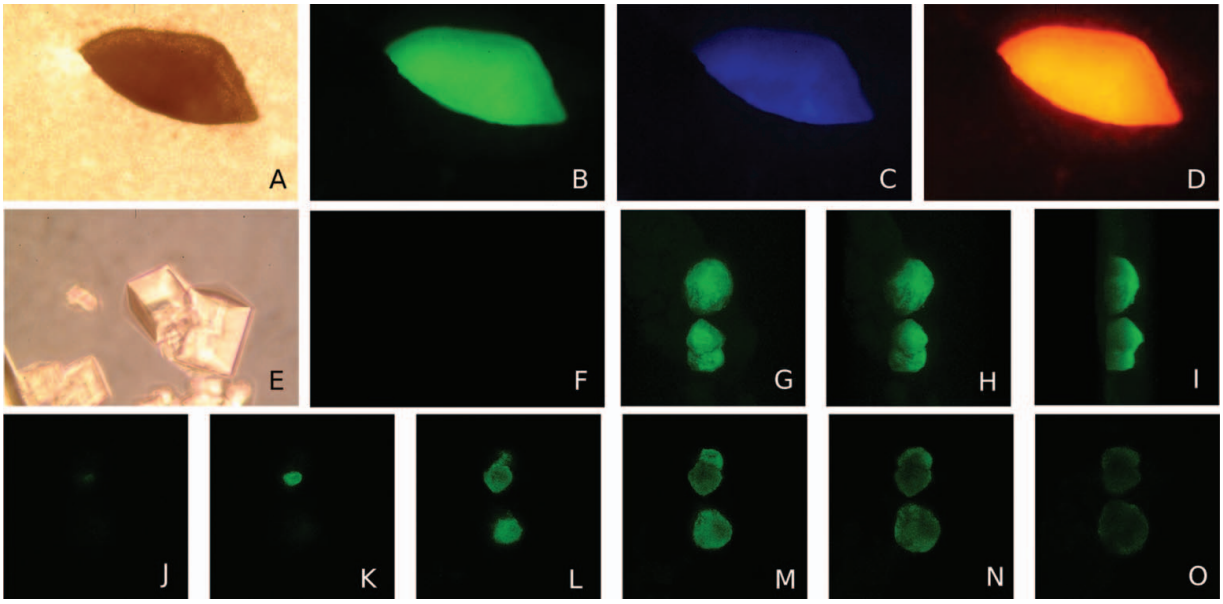

FIG. 5. $CaCO_3$ crystals precipitated by *B. subtilis* 168. Panels A–F, Crystals (magnification 100X) observed by fluorescent microscopy using the Fluorescein filter, no fluorescence (A), 480 nm (B), CY3 535 nm (C) and Dapi 360 nm (D). This fluorescence is absent in $CaCO_3$ crystals produced through forced precipitation (panels E and F). Panels G–O, Crystals (magnification 100X) observed by confocal microscopy. Panels G through I show a three-dimensional image (side to side) of two adjacent $CaCO_3$ crystals produced by *B. subtilis*. Panels J–O depict cross-sections from top to bottom using the Fluorescein filter. As shown by the green fluorescence, the organic matrix was part of the internal structure of the crystal (color figure available online).

of $CaCO_3$ (Visscher and Stolz 2005). The SI is defined as $SI = \log(IAP/K_{sp})$, where IAP denotes the ion activity product (i.e., $\{Ca^{2+}\}\{CO_3^{2-}\}$) and K_{sp} the solubility product constant of the corresponding mineral (Stumm and Morgan 1996). If $IAP > K_{sp}$, then the solution is oversaturated. Previous *in vitro* experiments showed that an $SI \geq 0.8$ is required before precipitation occurs (Kempe and Kazmierczak 1994).

Nucleation is another important factor for mineralization and may be affected by the extracellular organic matrix (comprised mainly of EPS). EPS has the capacity to bind cations (metals), including Ca^{2+} (Braissant et al. 2003; Dupraz et al. 2009a; Dupraz and Visscher 2005). Initially this complexation inhibits precipitation by reducing free calcium ions, but heterotrophic microbial degradation of EPS releases the bound calcium while simultaneously producing carbonate alkalinity (HCO_3^-), which can also enhance mineral precipitation (increase in SI) if the pH is high enough (Baumgartner et al. 2006; Dupraz et al. 2009a; Visscher and Stolz 2005).

Cation reactive organic polymers such as the EPS matrix can also affect the form of resulting crystals by binding to crystal growth sites, thus altering crystal growth rates (Benzerara et al. 2006; Morse et al. 2007). For example, calcium carbonate crystals isolated by Benzerara et al. (2006) from the environment were surrounded by a thick amorphous mineral layer embedded in an organic matrix, which resulted in unusual sizes and shapes of the crystals. Such results suggest that microbial organisms significantly impacted the mineralogy of carbonate (Benzerara et al. 2006).

Carbonate growing within EPS lacks cleavage and angular surfaces (Fig. 5). Our studies confirm these results on B4: globular and porous shapes are obtained with biologically precipitated crystals (Figs. 2 and 4). Examination of our crystals by scanning electron microscopy (SEM) reveals casts of bacteria clearly revealed on the surface of a calcium carbonate crystal (Fig. 2, strain B2). Trace amounts of phosphorus were also detected by EDS (Fig. 2, strains B2 and D2), and while these could be evidence of phosphate mineral artifacts, they could also be traces of cellular material (lysed or whole cells), phosphate associated with EPS or carry-over from the medium.

Nucleation can also be affected by reaction of calcium with cell surfaces (Bosak and Newman 2005). Gram negative cell surfaces contain layers shown to be reactive with calcium (Smarda et al. 2002) and studies using scanning transmission X-ray microscopy (STXM) also show reactivity of cell surfaces with calcium (Benzerara et al. 2004). Our results do not show any correlation of precipitation potential with Gram type indicating either that buffer chemistry was a powerful overriding factor to a more subtle nucleation effect, and/or that EPS production by the cell influences it. In fact EPS production has been proposed as a bacterial survival strategy to avoid entombment by mineral precipitation (e.g., Bontognali et al. 2008). Recently Martinez et al. (2010) proposed another mechanism for avoiding entombment in cyanobacteria, based on the metabolic maintenance of a positive surface charge at alkaline pH, reducing calcium complexation and the formation of nucleation sites.

Because pH affects the SI and is a key factor for organomineralization, we tested whether pH was at the origin of the precipitation potential of our different environmental isolates. Forty-nine different strains were isolated from different soils and classified according to colony morphology and

chromatogenesis, cell Gram type, and bacterial shape (Table S1). None of these attributes were correlated with $CaCO_3$ precipitation *in vitro*. However, the pH of the B4 precipitating medium appeared to be one of the most important factor needed for precipitation. In our experiments, precipitation was associated with increased alkalinity of the medium by the growth/metabolism of the isolates. No precipitation occurred when plates had a low (acidic) pH following growth. Buffering of B4 alters the alkalinity by subtracting H^+ or OH^- from the environment, which is reflected in the IAP. However, in our study, the "non-precipitating" strains could be forced to precipitate by buffering the medium at pH 8.2. Conversely, precipitating strains could be impaired in crystal formation by buffering the medium at pH 7.2. Thus, *in vitro* organomineralization is not only a property of the bacterial biofilm but is highly influenced by medium chemistry.

As previously discussed, buffering of the medium affects mineral precipitation. For example, environmental isolates grown on standard B4 medium may not precipitate initially, but may do so if they are transferred to grow in B4 medium buffered at a high pH. B4 is a very effective precipitation medium and consequently we analyzed why B4 holds these properties, by studying of the only buffered component in B4: yeast extract, which is rich in proteins. The titration revealed that there was a poor buffering capability between pH 6.5 and 7.5, bracketing the working pH range of sterile standard B4 (6.87 ($\pm$0.05)) (Fig. 3). In this range (from pH 6.5 to 7.5), a small variation of the proton concentration rapidly affects the pH of the medium, and consequently the precipitation. More studies are needed to clarify this aspect, such as experiments with fully defined media.

In conclusion, we obtained many different crystal morphologies using the different strains of bacteria. Results were consistent with calcium carbonate based on EDS analysis and dissolution with acid and were not correlated with any particular bacterial phenotype, but were highly dependent on medium pH. Although investigation of crystal type is important for interpretation of biomineralization influences, closer examination of these effects is beyond the scope of this particular study. For our purposes, the mere presence or absence of calcium carbonate minerals is sufficient to justify our conclusions about the efficacy of B4 medium for biomineralization studies.

Although B4 medium is used extensively to study the carbonate organomineralization potential of bacteria isolated from the environment (Baskar et al. 2006; Boquet et al. 1973; Urzì et al. 1999), our findings show that B4 provides only a partial indication of bacterial organomineralization potential. This is because changing of the buffering conditions also affects the precipitation. In other words, we conclude that a bacterial strain that is able to precipitate under environmental conditions can be unable to precipitate on B4 medium or *vice versa.* A good alternative might be to design a series of media varying only in one or few components, as recently reported by Sánchez-Román et al. (2011).

Such series can mimic different micro conditions and fluctuations that can be encountered in the microenvironment. Another alternative might be to use a fully defined medium. It is important to consider the buffering capacity of natural precipitating environments and not assume that an unbuffered to poorly buffered laboratory system based on B4 medium can be used as a predictive test for organomineralization potential. An approach using a defined medium designed to mimic the natural environment as closely as possible, including both pH and buffering potential, may be required to adequately determine which bacteria enhance mineral precipitation.

SUPPORTING INFORMATION

Crystal formation and strains characterization by environmental isolates and coloration of the plates after one and two weeks of incubation at 39°C. (R) pH indicator change to red after 1 or 2 weeks of incubation; (Y) yellow; (O) orange; (R) red. (+) crystals precipitation; (−) crystal precipitation does not occur. (*ng*) no growth. Matrix presence (Fig. 4) is indicated by *yes* or *no*. *B. subtilis* strain 168 was used as positive control and FBC5 (*etfA* mutant) as negative control.

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