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Precipitation of calcium carbonate by *Vibrio* spp. from an inland saltern

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Abstract: Calcium carbonate precipitation by 63 strains of moderately halophilic bacteria (*Vibrio* spp.) isolated from water of a saltern pond has been studied, taking into account the influence of salinity and temperature on the quantity and type of crystals precipitated. The bacteria formed crystals under all the conditions tested. All the crystals were magnesium calcite, with a variable Mg content, depending upon the medium provided. No aragonite was detected, even in a media with high Mg contents. Whether these microorganisms play an active role in precipitation is discussed, as well as the possibility that they may contribute to $CaCO_3$ precipitation in their natural habitats.

Key words: Hypersaline habitat; Calcium carbonate; *Vibrio*, Halophilic bacteria

Introduction

The role of bacteria in $CaCO_3$ precipitation has been known since the beginning of this century, and numerous authors have described $CaCO_3$ formation by bacteria of various taxonomic groups [1–3]. According to Boquet et al. [4], $CaCO_3$ precipitation is a general phenomenon in the bacterial world and under appropriate conditions all types of bacteria are capable of forming $CaCO_3$ crystals.

Soils, freshwater and saline habitats are natural environments where $CaCO_3$ precipitation by bacteria has been reported, and this microbial capacity has been related to the formation of marine calcareous skeletons, carbonate sediments, soil carbonates and carbonate rocks. Nevertheless, the exact role of the bacteria in the precipitation process is still not fully understood [5–8]. Both active and passive roles have been proposed for the bacteria: microbial bodies may act as crystal nuclei, seawater chemistry may be altered, calcium concentrated, and bicarbonate ions produced. Erlich [9] summarizes five possible mechanisms for the bacterial precipitation of calcium or calcium-magnesium carbonates: (1) aero-

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bic or anaerobic oxidation of carbon compounds devoid of nitrogen in neutral or alkaline environments with a supply of calcium or magnesium; (2) aerobic or anaerobic oxidation of organic nitrogen compounds in unbuffered environments with a supply of calcium or magnesium; (3) $CaSO_4$ reduction by sulfate reducing bacteria; (4) the hydrolysis of urea; and (5) photosynthesis.

Some environmental characteristics could affect the precipitation of $CaCO_3$; among these the ionic strength of the medium may be the most important factor [10,11]. Nevertheless, studies into the influence of this factor are rare, owing to the fact that most microorganisms cannot grow at high osmolarities.

Moderately halophilic bacteria, which can grow over a wide range of osmotic concentrations [12], constitute an extremely useful group of microorganisms in which the influence of the ionic strength of the medium on biological $CaCO_3$ precipitation can be investigated.

A study of the metabolic activity of microorganisms isolated from hypersaline habitats provides clues to their potential activity in nature [13]. Some moderately halophilic bacteria have previously been reported to be capable of forming $CaCO_3$ precipitates [14–17]. The results of these studies suggest that different species differ in their response to increasing salinity not only by varying the crystalline type of $CaCO_3$ formed, but also in the extent of crystal formation. A confirmation of these different responses could help us to understand more fully the role that bacteria play in calcium carbonate precipitation in nature.

As part of a wider study we are investigating different strains of moderately halophilic microorganisms and their capacity to precipitate calcium carbonate. In this paper we report on 63 strains of moderately halophilic Gram-negative rods of the genus *Vibrio* [19] isolated from water samples. The aim of our work has been: (1) to investigate the ability of moderately halophilic *Vibrio* spp. to precipitate carbonate compounds, taking into account the influence of certain external factors such as salt concentration and incubation temperature on this precipitation; and (2) to make a mineralogical study into the type of carbonate formed and the morphology and composition of the crystals.

We also discuss the involvement of *Vibrio* spp. in carbonate precipitation in their natural habitats.

Materials and Methods

Microorganisms

The 63 bacterial strains selected for this study were isolated from water samples taken from ponds of a saltern that has been described in ecological terms in a previous study [18]. They were identified and assigned to the genus *Vibrio* [19].

Culture media

Isolates were grown on the MH medium described by Quesada et al. [20], composed of (g/l): yeast extract (Difco), 10; proteose-peptone no. 3 (Difco), 5; glucose, 1; calcium acetate, 4, and supplemented with a balanced mixture of sea salts [21] prepared at 30% w/v and diluted to give final concentrations ranging from 2.5% to 20% (w/v). The stock sea-salt solution (30%, w/v) had the following composition (g/l): NaCl, 234; $MgCl.6H_2O$, 41.6; $MgSO_4.7H_2O$, 59.82; $CaCl_2 2H_2O$, 1.10; KCl, 6; $NaHCO_3$, 0.2; NaBr, 0.7; $FeCl_3.6H_2O$, trace. This medium was solidified with 20 g/l Bacto-Agar (Difco). The pH was adjusted to 7.2 with 1 M KOH.

Saline water analysis

The Ca^{2+} and Mg^{2+} concentrations in the saline water from which the bacterial strains were isolated were determined by atomic absorption spectrophotometry (Pyeumcam, model SP9OA). The pH was measured directly with a standard pH-meter (Radiometer Copenhagen PHM62), equipped with a glass electrode.

Studies of crystal formation

The 63 strains were surface-inoculated onto solid media at 2.5%, 7.5%, 15% and 20% (w/v) salt concentration. The plates were examined periodically up to 25 days after inoculation by opti-

cal microscopy for the presence of crystals. These experiments were carried out in triplicate at 22°C and 32°C. In all experiments, controls were included consisting of uninoculated culture media and media inoculated with autoclaved bacterial cells.

Analysis of crystals

Two *Vibrio* strains (A-11 and type strain A-32), selected on the basis of their higher crystal yield, were plated on media containing 2.5%, 5%, 7.5%, 10%, 12.5%, 15%, 17.5% and 20% (w/v) total salt concentration. Crystals formed by the selected strains were removed from the medium by cutting out agar blocks and placing them in a boiling water bath (5 ml) until the agar dissolved. The supernatants were decanted, and the sediments resuspended and washed in distilled water until the crystals were free of impurities. The washed crystals were air-dried at 37°C.

The purified crystals were examined by X-ray diffraction (XRD) (powder diagrams) with Philips Pw 1140 and Rigaku-Miniflex Ca 2005 instruments, equipped with a nickel filter and CuKα radiation, and identified according to J.C.P.D.S. and A.S.T.M. criteria [22,23]. The diffraction peak corresponding to planes H4 3 104 (d approx. 3 Å) was used to determine the approximate magnesium content of the calcite [24]. Quartz Fisher was added to the samples as a standard for calibration of the diffractometer.

Purified crystals were also analyzed with an Eppendorf flame emission spectrometer (FS) to determine calcium content, after being digested with dilute hydrochloric acid (HCl 1:1 v/v).

The morphology of the crystals was studied in a Hitachi S- 510 scanning electron microscope, using gold metallization.

The calcium and magnesium contents of the crystals was determined with an X-ray microanalyzer EDAX-9900 (EDX) coupled to a Philips 505 S.E.M. Experimental conditions were as follows: pin-point analysis, 10000×, 18 Kv, spot size 50–100 nm, tilt angle 35° and rate 1000 graphite coating was used. Calcite ($CaCO_3$) and dolomite ($Ca_{0.5}$ $Mg_{0.5}$ CO_3) of high purity from the Betic mountains were used as standards. The

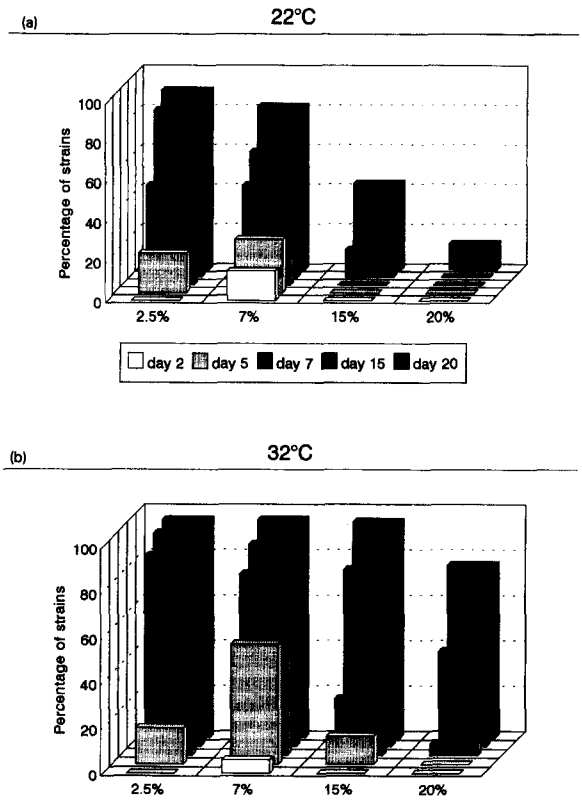

Fig. 1. Percentage of calcium carbonate precipitating strains of *Vibrio* with time at different salt concentrations (%, w/v) cultured at 22°C (**a**) and 32°C (**b**).

results reported in this study are the average of five determinations.

Results and Discussion

The percentage of *Vibrio* strains which caused the precipitation of $CaCO_3$ and the time required for precipitation to take place at the different concentrations of total salts assayed (2.5%, 7.5%, 15% and 20% w/v) at both incubation temperatures (22°C and 32°C) are set out in Fig. 1. The formation of calcium carbonate crystals was detected under all the conditions tested, although the number of crystal-forming strains decreased concomitantly with an increase in salt content. No crystal formation was seen on the control plates.

Observation under a microscope showed that precipitation always occurred within colonies of bacteria, indicating that this precipitation takes place in a microenvironment provided by bacterial growth. Both the size and quantity of the crystals increased with time. At 2.5% salt concentration many large crystals developed; a very slight diminution was to be seen at 7.5%, whilst at 15% and particularly 20% very few, smaller crystals were detected.

The strains of *Vibrio* studied grow best at 7.5% salt solution [19] and so the concentration for optimum carbonate precipitation (2.5%) does not appear to coincide with that for optimum growth. With regard to this fact, Rivadeneyra et al. [11] have reported that Mg^{2+} has an inhibitory effect on carbonate precipitation by bacteria. This ion was present in the culture media and it may be presumed that increasing concentrations of it was one of the factors contributing to the diminution in crystal precipitation in these experiments. Nevertheless, the slight difference in crystal formation between 2.5% and 7.5% salt concentration leads us to believe that the inhibitory effect of Mg^{2+} is significantly less with moderately halophilic bacteria than with non-halophilic ones as Ferrer et al. [15] have indicated elsewhere.

As far as the incubation temperature is concerned, the number of crystal-forming strains remains higher at 32°C than at 22°C (Fig. 1). At 2.5% and 7.5% the differences in crystal production are negligible and may be put down to the fact that 32°C is the optimum growth temperature for these bacteria. Nevertheless, we believe that other reasons should be sought to explain the increased performance observed at higher concentrations (Fig. 1). Morita [25] and Ferrer et al. [15,16] affirm that high temperatures favour the precipitation of $CaCO_3$, but Rivadeneyra et al. [17] say that incubation temperature only influences the formation of calcium carbonate when external conditions are not ideal for its precipitation. Thus, at high Mg^{2+} concentrations, $CaCO_3$ precipitation becomes more difficult and temperature may play an important part in its formation, whereas at low Mg^{2+} concentrations, which are more suitable for its precipitation, temperature would have less effect.

It has been reported [15,26,27] that both temperature and external salt concentrations produce important changes in the internal composition and physiological activity of moderately halophilic bacteria. Our results show that these two factors greatly influence crystal precipitation by these bacteria. Table 1 sets out the results of studies by X-ray diffraction and flame spectrophotometry of the crystals obtained at different salt concentrations. Mg^{2+} concentrations and Mg^{2+}/Ca^{2+} ratios in the media are also indicated. Both techniques always indicated that magnesium calcite had been formed. The Mg^{2+} content of these calcites increased concomitantly with an increase in total salinity and the Mg^{2+}/Ca^{2+} ratio (Fig. 2). The incubation temperature had no effect upon the composition of the crystals.

Table 1

Results of crystal analysis (XRD and FS) obtained in media at different salt concentrations

% Salt *	Mg^{2+} (ppm) *	Mg/Ca (molar relation *	Crystalline species	d(104)Å	Formulation (XRD)	Formulation (FS)
2.5	906	1.5	magnesium calcite	3.001	$Ca_{0.88}\ Mg_{0.12}\ CO_3$	$Ca_{0.85}\ Mg_{0.15}\ CO_3$
5	1812	2.8	magnesium calcite	2.976	$Ca_{0.79}\ Mg_{0.21}\ CO_3$	$Ca_{0.77}\ Mg_{0.23}\ CO_3$
7.5	2717	4.1	magnesium calcite	2.965	$Ca_{0.76}\ Mg_{0.24}\ CO_3$	$Ca_{0.72}\ Mg_{0.28}\ CO_3$
10	3623	5.4	magnesium calcite	2.963	$Ca_{0.75}\ Mg_{0.25}\ CO_3$	$Ca_{0.70}\ Mg_{0.30}\ CO_3$
12.5	4529	6.6	magnesium calcite	2.950	$Ca_{0.70}\ Mg_{0.30}\ CO_3$	$Ca_{0.68}\ Mg_{0.32}\ CO_3$
15	5435	7.7	magnesium calcite	2.929	$Ca_{0.65}\ Mg_{0.35}\ CO_3$	$Ca_{0.63}\ Mg_{0.37}\ CO_3$
17.5	6341	8.8	magnesium calcite	2.935	$Ca_{0.64}\ Mg_{0.36}\ CO_3$	$Ca_{0.64}\ Mg_{0.36}\ CO_3$
20	7247	9.8	magnesium calcite	2.931	$Ca_{0.63}\ Mg_{0.37}\ CO_3$	$Ca_{0.63}\ Mg_{0.37}\ CO_3$

* Culture medium.

Studies carried out into the inorganic precipitation of carbonates in hypersaline environments [28] and also spelean carbonates [29] have shown that, when the Mg^{2+}/Ca^{2+} ratio in the medium increases, the Mg^{2+} content in the magnesium calcite does so as well, although when the Mg^{2+}/Ca^{2+} ratio becomes higher than 1 [29] or 2 [28] both calcite and aragonite are produced. Other authors [30–34] have concluded that Mg^{2+} ions inhibit calcite precipitation but enhance that of aragonite. The concentration of Mg^{2+} ions in our media ranged from 906 to 7.247 ppm and the molar Mg/Ca ratio was about 1. 5 in the 2.5% salt medium and 9.8 at 20% total salts. Under these conditions aragonite might be expected to precipitate, and, in fact, the formation of aragonite by certain species of *Vibrio* isolated from seawater and grown in liquid media has been reported [1,2,35]. We, on the other hand, detected no aragonite in any of our cultures.

Our results seem to indicate that the mechanisms employed by moderately halophilic bacteria to precipitate calcium carbonate may well differ from those of non-halophilic species of *Vibrio* and also those involved in its inorganic deposition. We believe, as do other authors [16,17,27] that moderately halophilic bacteria play an active role in the precipitation of the calcium carbonate and that it is not just deposited as an indirect consequence of environmental changes brought about by their metabolic activity.

The results of S.E.M. observations are shown in Fig. 3. The carbonate precipitates in the form of globular crystal aggregates. Sometimes these bioliths are coated with a thin mineral layer (Fig. 3d) and sometimes not (Fig. 3a). The reason for this is not yet fully understood but it may be related to the stage of formation of the globule. Their internal structure is one of a large number of radially orientated canaliculi (Fig. 3c,e). When the globules are uncoated these canaliculi reach the surface in the shape of pores approx. 0.1 to 0.2 mm in diameter (Fig. 3b). The cores of the globules consist of a large pore (Fig. 3e). The globules exist individually or may be welded together by carbonate cement (Fig. 3f). This morphology is consistent with that of carbonates produced by other moderately halophilic bacteria

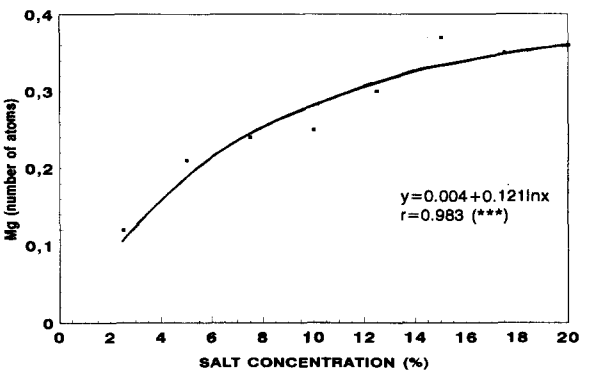

Fig. 2. Magnesium content of crystals precipitated in media of different salt concentrations (%, w/v).

[15,16] and thus seems to indicate that the precipitation of such crystals by bacteria in media with a high Mg^{2+} content tends to produce these shapes.

The results of EDX analysis are shown in the caption to Fig. 3. They confirm the presence of magnesium calcite. The Mg^{2+} content is lower in the nucleus and increases progressively towards the edges of the globules. This Mg^{2+} zoning within the globules coincides with results obtained by Arnauld and Herbillon [36] in accumulation layers and concretions of magnesium calcites found in various soils, which they attribute to "partitioning effect precipitation" mechanisms. We have observed a decrease in Mg^{2+} in the globule coats, however, which cannot be explained by these mechanisms.

Some authors have studied the involvement of microorganisms in calcite precipitation in natural hypersaline environments [37,38]. *Vibrio* spp. have been isolated from an inland saltern at la Malá (Granada), the ecological aspects of which have been described elsewhere [18]. Water analyses from this site gave the following results: Ca^{2+} 3.600 ppm and Mg^{2+} 1.300 ppm. The pH ranged from 7.5 to 8.7. These physico-chemical characteristics of the natural habitat of *Vibrio* are more suitable for the precipitation of carbonates than are those of our culture media, i.e. the Mg^{2+} is lower in the natural habitat, except for our 2.5% salt solution, which has only 906 ppm Mg^{2+}. Thus we have good reason to believe that, although our results were obtained in vitro, the species we

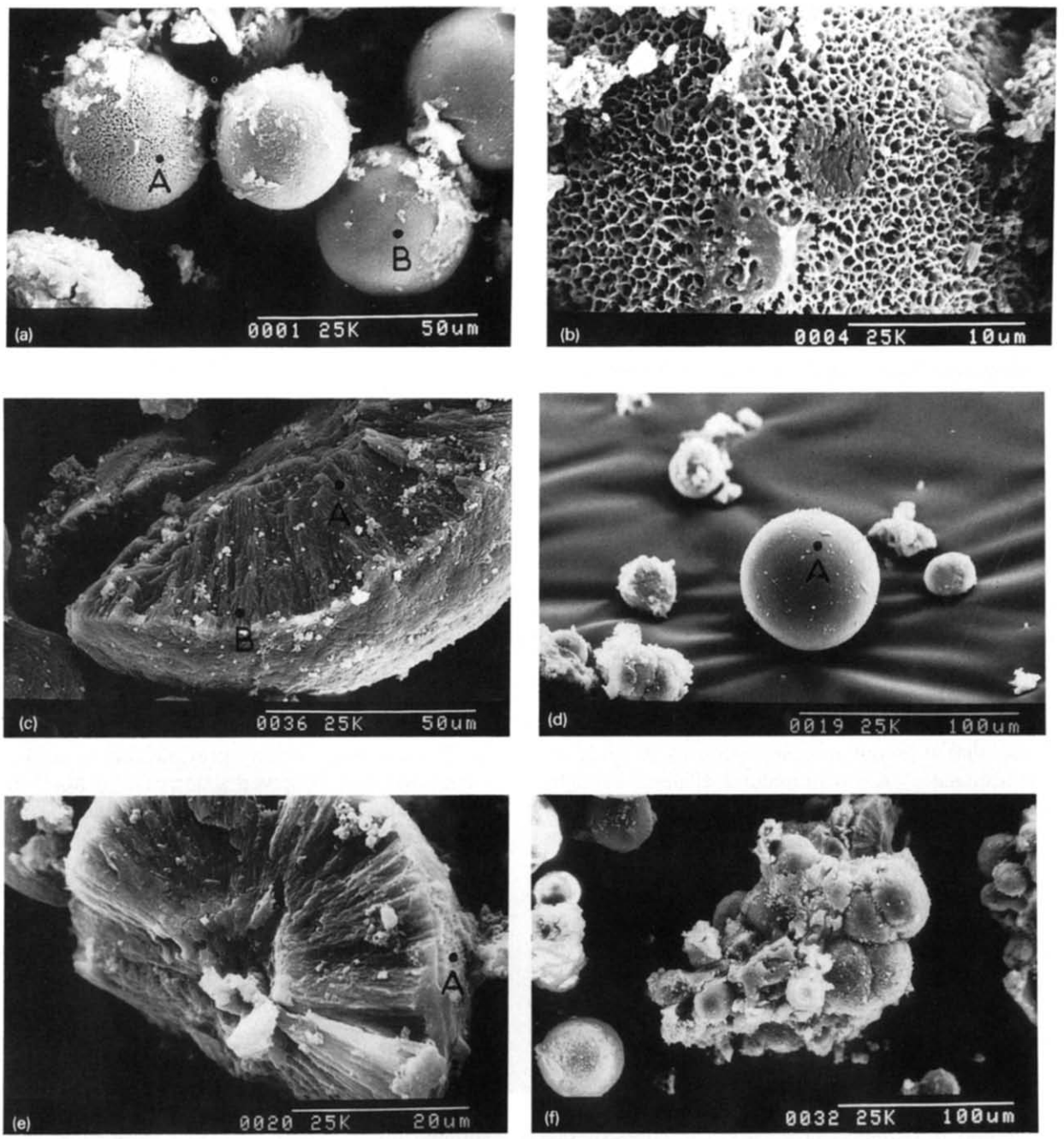

Fig. 3. Scanning electron microscopy and EDX results. **(a)** Photo 0001: 2.5% (w/v) salinity. Globular forms covered and uncovered. Formula by EDX: A = $Ca_{0.92}$ $Mg_{0.08}$ CO_3; B = $Ca_{0.80}$ $Mg_{0.20}$ CO_3. **(b)** Photo 0004 2.5% (w/v) salinity. Porous surface. **(c)** Photo 0036: 2.5% (w/v) salinity. Fibrous radiated internal estructure. Formula by EDX: A = $Ca_{0.84}$ $Mg_{0.16}$ CO_3; B = $Ca_{0.93}$ $Mg_{0.07}$ CO_3. **(d)** Photo 0019: 20% (w/v) salinity. Globular form covered. Formula by EDX: A = $Ca_{0.56}$ $Mg_{0.44}$ CO_3. **(e)** Photo 0020: 20% salinity. Fibrous radiated internal structure and central void, uncovered form. Formula by EDX: A = $Ca_{0.61}$ $Mg_{0.39}$ CO_3. **(f)** Photo 0032: 20% (w/v) salinity. Aggregated globules.

have tested are capable of causing the precipitation of $CaCO_3$ in their natural habitat in the same way as they did in the laboratory. Furthermore, the frequency of these bacteria in their natural saline habitat combined with the abundance of calcite deposits supports our view that they play an important role in these deposits. More exhaustive studies are still required, however, to determine the exact role the bacteria play and their contribution to the precipitation of calcites in hypersaline environments.

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