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Precipitation of Calcium Carbonate by *Deleya halophila* in Media Containing NaCl as Sole Salt

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Abstract. The precipitation of calcium carbonate by 27 strains of *Deleya halophila* using solid and liquid media containing different NaCl concentrations (2.5, 7.5, or 20%, wt/vol) as sole salt, and two incubation temperatures (22° and 32°C) have been studied. All the strains tested were able to precipitate calcium carbonate under the different environmental conditions assayed. Crystals formed were calcite and vaterite; the ratio of calcite to vaterite was dependent on total salts and on the type of medium.

Since the first reports that bacteria could precipitate calcium carbonate ($CaCO_3$) were presented, numerous authors have made reference to the extracellular precipitation of $CaCO_3$ and other insoluble carbonates. These precipitations are carried out by different groups of bacteria under diverse environmental conditions [6, 13, 19–21, 27].

The complete mechanism of $CaCO_3$ precipitation by microorganisms is not understood in all cases, although it always depends on metabolic CO_2 production or consumption and on the relative solubility of $CaCO_3$ [10].

Microbial precipitation or $CaCO_3$ has been demonstrated in soils, freshwaters, and marine environments, and it has been suggested that microbial activity could be related to the formation of marine calcareous skeletons, carbonate sediments, and carbonate rocks [1, 5, 8, 18].

Some environmental characteristics can affect the precipitation of $CaCO_3$; among them, ionic strength of the medium may be the most important factor. Nevertheless, investigations into the influence of this factor are scarce, owing to the fact that most microorganisms cannot grow at high osmolarities, and only some of them grow optimally over a wide range of salt concentrations.

Deleya halophila is a new, moderately halophilic species, isolated from a saline soil located near Alicante (Spain) [22]. The bacterium is a Gram-negative non-sporeforming motile rod, and it is aerobic, having a strictly respiratory type of metabolism. It has been classified as a moderate halophile, since it grows optimally at 7.5% (wt/vol) salts. *Deleya halophila* also exhibits a strong euryhaline character, since it is able to grow between 2 and 25% (wt/vol) salts. It has an absolute requirement for the sodium cation, which is not replaced by other cations, or sucrose. In contrast with other halophilic species, *D. halophila* can grow in media without added Mg^{2+} or K^+ [23].

The growth characteristics and salt requirement of *D. halophila* may be useful in studies on $CaCO_3$ precipitation, since it offers the possibility of studying the influence of a wide range of different ionic strengths on the process.

In a previous study [11], we investigated the role of salt concentrations in $CaCO_3$ precipitation by this bacterium. Our results indicated that the optimal salt concentration for this process was 2.5% (wt/vol), whereas a higher salt concentration (20%, wt/vol) precipitation was strongly inhibited. These results confirmed the observations made by Billy [4], who found that excess salts negatively influenced $CaCO_3$ crystal formation. Nevertheless, we did not know whether the inhibitory effect of high salt concentration was due to the great magnesium content in the salt solution used, since a negative effect of magnesium on calcium precipitation by other microorganisms was reported by Rivadeneyra et al. [24].

In this investigation, we have studied $CaCO_3$ precipitation by 27 strains of *D. halophila*, using media with different NaCl concentrations (2.5, 7.5, and 20%, wt/vol) and two incubation temperatures, 22° and 32°C respectively. The ability of different

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strains to form crystals, the extent of precipitation, and the type of crystal formed have been investigated.

Materials and Methods

Strains. All 27 strains selected for this study were moderately halophilic rods, classified as *Deleya halophila* [22] and isolated from hypersaline soils. The type strain of this species (CCM 3662 = strain F5-7) was also included.

Culture media. The media contained (g/L): yeast extract (Difco), 10; proteose-peptone no. 3 (Difco), 5; glucose, 1; calcium acetate, 4; supplemented with NaCl to give final concentrations of 2.5%, 7.5%, or 20% (wt/vol). The pH values were adjusted to 7.2 with 1 mol/L KOH. To obtain solid media, 20 g/L Bacto-Agar (Difco) was added.

Studies on crystal formation with solid media. The bacteria were plated on media containing the three different NaCl concentrations described above. The plates were examined periodically up to 25 days after inoculation by optical microscopy for the presence of crystals. These experiments were carried out in triplicate at 22° and 32°C. In all the experiments, controls were included consisting of uninoculated culture media and media inoculated with autoclaved bacterial cells.

For the analytical studies, crystals formed by the type strain (CCM 3662) were removed from the medium by cutting out agar blocks and placing them in a boiling water bath until the agar dissolved. The supernatants were decanted, and the sediments were resuspended and washed in distilled water until crystals were free of impurities. Finally, the washed crystals were air-dried at 37°C.

Studies on crystal formation with liquid media. Liquid media containing NaCl concentrations identical with those of the solid media were used (100 ml of medium into 250 ml Erlenmeyer flasks). After 25 days of incubation at each temperature, crystals formed by strain CCM 3662 were collected from the liquid medium surface, bottom, or adherent to the walls. Then, they were transferred into distilled water, purified, and dried, as described above.

Analysis of crystals. The purified crystals were examined by X-ray diffraction (powder diagram) using a Rigaku-Miniflex Ca 2005 instrument equipped with a proportional counter (CM5757, D1, SPC-20), gas detector, nickel filter, and Cukα-radiation. Preparation of the samples involved pulverizing purified crystals in a agate mortar before analysis.

Crystal identification was carried out according to the Joint Committee on Powder Diffraction Standards Criteria [2, 3]. Compositional semiquantitative analysis of crystals was performed following the intensity factors method [17, 26]. The estimated diffraction peak in calcite was 104 (d = 3.035 Å) and that of vaterite was 112 (d = 2.730 Å).

Morphological study of crystals. Purified crystals were examined in a Hitachi S-510 Scanning Electronic Microscope (SEM), operated at 25 KV. The samples were coated with gold.

Results

Figure 1 shows the percentages of strains of *Deleya halophila* that precipitated $CaCO_3$ and the time required for precipitation at the three different salt concentrations and two incubation temperatures. No differences between crystal formation on solid and in liquid media were found; Fig. 1 shows the results obtained with solid media. Control plates and tubes were free of crystals in all cases.

Table 1. Composition of crystals formed by *Deleya halophila* CCM 3662^T

Media	Polymorph (%)	NaCl concentration (%, wt/vol) 2.5	7.5	20
Solid	Calcite	>99	96	93
	Vaterite	Traces	4	7
Liquid	Calcite	>99	81	49
	Vaterite	Traces	19	51

Microscopic observations showed a high amount of crystals formed under all environmental conditions tested, although we noted a slight decrease of precipitate at 20% (wt/vol) NaCl; moreover, the crystal sizes at this salt concentration were small than at 2.5 or 7.5% (wt/vol) NaCl.

X-ray studies demonstrated that the crystals formed by *D. halophila* CCM 3662 were calcite and vaterite. Percentages of both crystals formed at the different NaCl concentrations on solid media and in liquid media are shown in Table 1. As can be seen, the ratio of calcite/vaterite was dependent on total salt and on the type of medium.

In Fig. 2, the morphologies of crystals produced by the type strain, as detected by SEM, are shown. Crystals formed on both solid and liquid media under the different conditions showed similar morphologies. The most abundant were globular or quasispherical forms with sizes ranging between 50 and 100 μm in diameter. These crystals were preferentially single and rarely linked by carbonated substances of attachment. Their surfaces, in the majority of cases, were rugose with great porosities, althogh some of the spherical forms appeared covered with a thin layer of carbonate, which gave them a smooth appearance (Fig. 2a). The greatest number of cases had internal fibrous-radiated structures, which can be observed in Fig. 2b.

In some of the samples, particularly in those samples taken from media containing 20% (wt/vol) NaCl, we recognized forms that possessed crystalline habits similar to orthorhombic prisms (Fig. 2c, 2d), whose surfaces showed cleavage systems or small crystals (Fig. 2e) with derivatized angles of 120° (hexagonal crystalline system). Inside, they

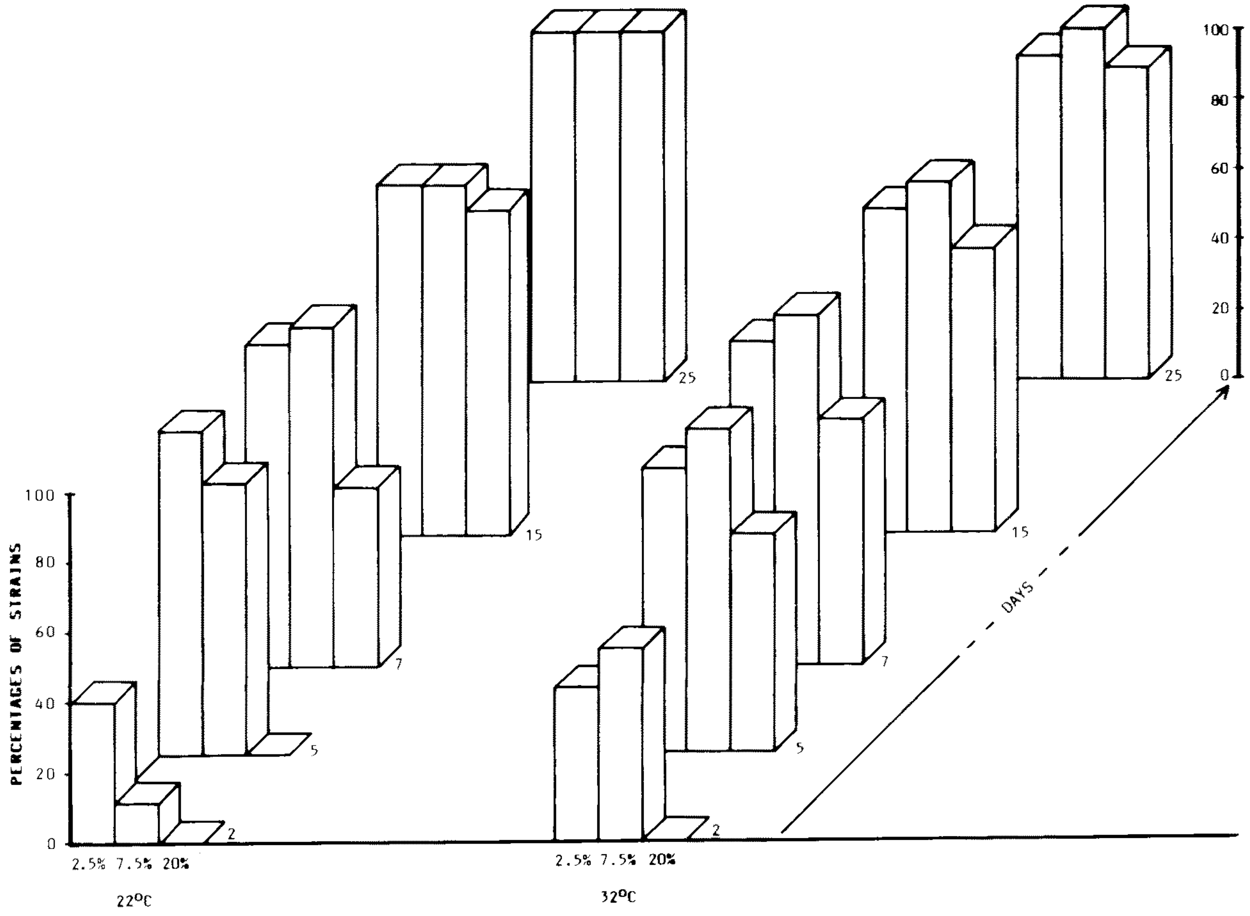

Fig. 1.

showed porous parts and other less porous portions, which constituted the core (Fig. 2f). Sometimes, these forms were grouped according to twin law (Fig. 2g). Between the globular forms and the orthorhombic prisms, there appeared to be intermediate forms, occurring as spherulites with vestiges of crystallographic faces (Fig. 2h).

Discussion

One of the physiological activities already described for some moderately halophilic bacteria is their capacity to precipitate $CaCO_3$. According to Billy [4], an excess of salts negatively influences $CaCO_3$ by these bacteria; however, as can be seen from Fig. 1, all strains of *Deleya halophila* studied here were able to form $CaCO_3$ crystals under all of the different conditions tested, if they were able to grow.

The extent of crystal formation was very high at all salt concentrations studied, although precipitation took place at different times, depending on the NaCl concentration and the incubation temperature. These observations can be related to the growth characteristics and salt requirement of *D. halophila* [23]. The bacteria grew optimally at 7.5% (wt/vol) salts at 32°C, with the highest growth rates (0.56 h^{-1}), but when they were cultured at 22°C, they grew better at lower salt concentrations (2.5–5%, wt/vol). Consequently, $CaCO_3$ precipitation was fastest at 7.5% (wt/vol) NaCl if the strains were cultured at 32°C, but took place fastest at 2.5% (wt/vol) NaCl when bacteria were incubated at 22°C. At 20% (wt/vol) NaCl, precipitation occurred later because the strains grew with lower growth rates at both incubation temperatures.

In previous works, with *D. halophila* and other moderately halophilic microorganisms, designated as *Acinetobacter* and *Flavobacterium* [11, 12], using media containing magnesium, the authors found that the salt concentration for optimal crystal formation (2.5%, wt/vol) did not coincide with that for optimal growth (7.5%, wt/vol). They inferred from these observations that an increase in ionic strength of the medium inhibited $CaCO_3$ precipitation. This was

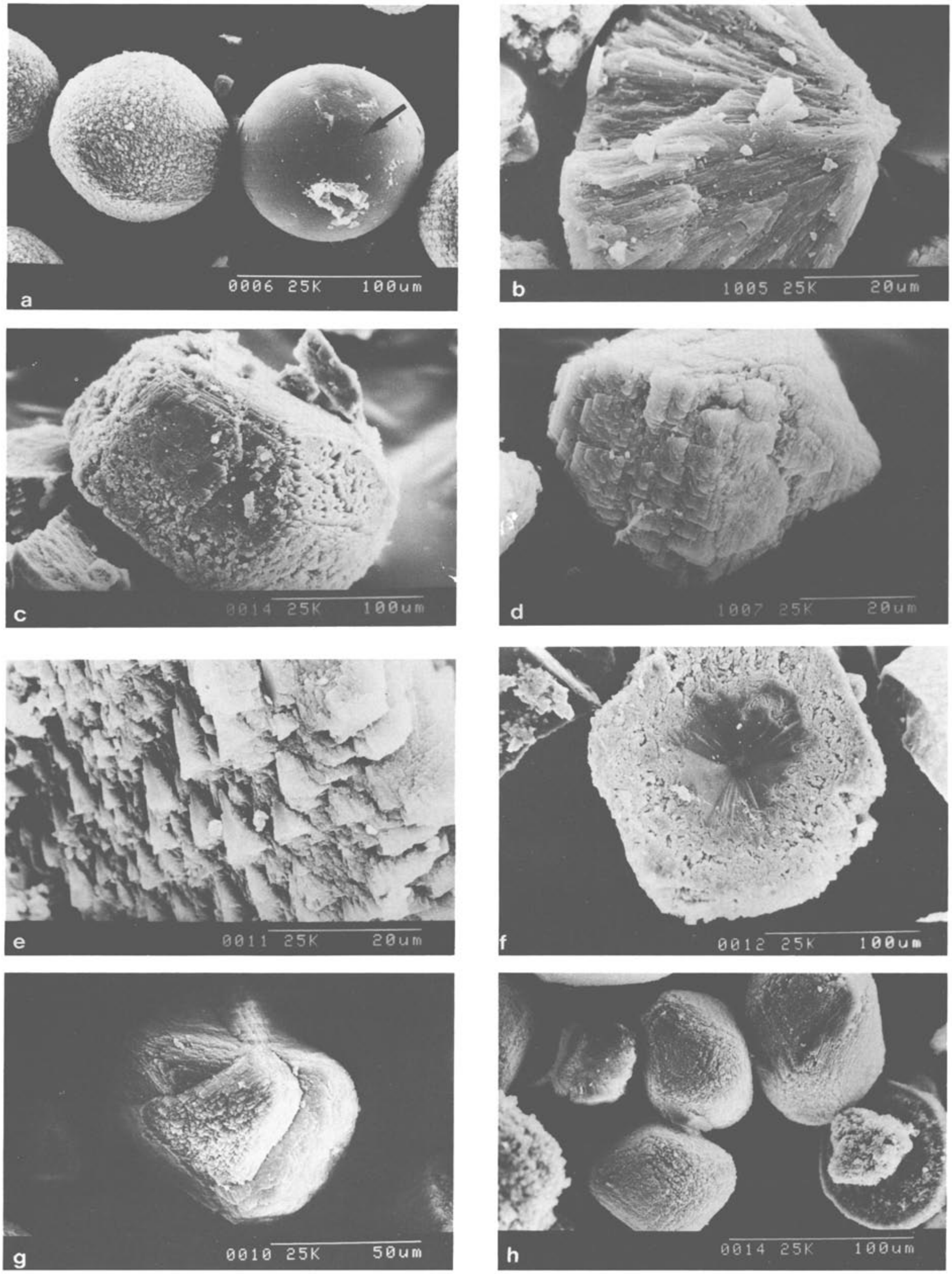
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Fig. 2.

also found by Billy [4]. Our results support the hypothesis that magnesium ions were responsible for the inhibitory effect described, since this cation increased in the culture medium as total salt concentrations were increased [11, 12].

Conversely, at 20% (wt/vol) NaCl, the extent of precipitation was slightly less and the crystals were smaller compared with those at 2.5 or 7.5% (wt/vol) NaCl. It is possible, therefore, that the inhibitory effects were due to Cl^-, and especially Na^+ ions, as on chemical (nonbiological) precipitation of $CaCO_3$, reported by Cailleau et al. [7]. However, the effect of high NaCl concentrations on $CaCO_3$ formation by *D. halophila* seems to be less marked than that described on chemical $CaCO_3$ precipitation by Cailleau et al. [7].

With respect to incubation temperature, we could not detect significant differences. This may be because all strains grew well at both temperatures (22° and 32°C). Other authors have reported that $CaCO_3$ precipitation was influenced by incubation temperature, with precipitation being greatest at high temperatures [11, 12, 20]. We believe that temperature exerts an indirect action because the bacterial growth is related to it, and it is suggested that when suitable conditions for $CaCO_3$ precipitation are present, the incubation temperature does not affect the process.

The results of crystal identification performed by X-ray diffraction techniques (Table 1) showed some differences in the type of crystal formed by *D. halophila*, depending on type of medium and salt concentration. At 2.5% (wt/vol) NaCl, *D. halophila* formed calcite and only traces of vaterite, whereas at 7.5 or 20% (wt/vol) NaCl, the bacteria precipitated calcite and vaterite. The highest amount of vaterite was formed at 20% (wt/vol) NaCl with liquid media (51%).

These results contrast with those of Cailleau et al. [7], who reported that vaterite and calcite were formed (chemical precipitation) in the range from 0 to 0.25% (wt/vol) NaCl, but over this value, only vaterite was precipitated. According to Kitano and Hood, Kitano et al. [15, 16], Cailleau et al. [7], Kamiya et al. [14], and Sayoko and Kitano [25], the presence of Mg^{2+} in the medium favors aragonite formation, while Rivadeneyra et al. [24] reported that this effect is less marked in bacterial precipitation. In the same way, Ferrer et al. [11, 12] described that *D. halophila* and *Flavobacterium* precipitated calcite as the sole precipitation form of $CaCO_3$, using media containing high concentrations of Mg^{2+}, whereas *Acinetobacter* formed calcite and traces of aragonite. From these results, it can be inferred that bacterial type plays an active role in both $CaCO_3$ precipitation and the form of its crystallization. Moreover, solid medium favored calcite formation, whereas liquid medium facilitated vaterite crystallization.

The morphological studies (SEM) demonstrated that the most common crystalline forms were spherical. The rest were polyedric forms, which develop towards spherical forms during growth, and aggregates of individual crystals (Fig. 2g, 2h).

We also detected a case of crystal pseudomorphism of forms with orthorhombic symmetry (Fig. 2c, 2d) in minerals included in the hexagonal symmetry system. This may be due to the existence of different phases in the crystallization process, and to polymorphic transformations: aragonite (orthorhombic polymorph) giving calcite and/or vaterite (hexagonal polymorphs). The last two polymorphs would continue to grow over the pseudomorphic crystals, changing their morphologies to spherical (Fig. 2h). This hypothesis is based on photomicrographs which showed that orthorhombical forms were most abundant in media with the highest NaCl concentration, where the crystallization process was slower. In the same way, Ducloix and Dupuis (9) studying $CaCO_3$ chemical precipitation in media with a high content of NaCl and organic compounds from soil humus (for example, fulvic acid) described an initial precipitation of aragonite, which eventually developed to calcite.

The results obtained here lead us to support the hypothesis made by Morita [20] and Ferrer et al. [12], who concluded that bacteria could play an active role in $CaCO_3$ precipitation, and that mineral formation resulted not only as an indirect consequence of environmental changes, produced by metabolic activities of bacteria. This suggestion is based essentially on two facts: (a) different bacteria precipitate different types of $CaCO_3$ polymorphs and/or different concentrations of them; (b) the presence of some ions exerts a distinct effect on chemical and biological precipitation, both concerning the mineral products obtained (in some cases different polymorphs are precipitated, and in other cases, different concentrations of them) and the intensity of the inhibitory effect, which is usually lower in bacterial than in chemical precipitation.

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