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Microbial fossilization in carbonate sediments: a result of the bacterial surface involvement in dolomite precipitation

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ABSTRACT

Recent dolomitic sediment samples from Lagoa Vermelha, Brazil, were examined microscopically to study the process of bacterial fossilization in carbonate sediments. Bacteria-like bodies were intimately associated with carbonate mineral surfaces, and coatings on the former demonstrate the calcification of single bacterial cells. The bacterial fossilization process in Lagoa Vermelha sediments was simulated in the laboratory by cultivation of mixed and pure cultures of sulphate-reducing bacteria, which were isolated from the Lagoa Vermelha sediments. These cultures produced carbonate minerals that were studied to provide insight into the initiation of the fossilization process. In mixed culture experiments, bacterial colonies became calcified, whereas in pure culture experiments, single bacterial cells were associated with dolomite surfaces. Dolomite nucleated exclusively in bacterial colonies, intimately associated with extracellular organic matter and bacterial cells. Electrophoretic mobility measurements of the bacterial cells in electrolyte solutions demonstrated the specific adsorption of Ca^{2+} and Mg^{2+} onto the cell surfaces, indicating the role of the bacterial surface in carbonate nucleation and bacterial fossilization. The affinity of the cells for Mg^{2+} was related to the capability of the strains to mediate dolomite formation. Combined with sulphate uptake, which dissociates the $[MgSO_4]^0$ ion pair and increases the Mg^{2+} availability, the concentration of Mg^{2+} ions in the microenvironment around the cells, where the conditions are favourable for dolomite precipitation, may be the key to overcome the kinetic barrier to dolomite formation. These results demonstrate that bacterial fossilization is a consequence of the cell surface involvement in carbonate precipitation, implying that fossilized bacterial bodies can be used as a tool to recognize microbially mediated carbonates.

Keywords Bacterial fossils, carbonates, cell surface, dolomite, fossilization, zetapotential.

INTRODUCTION

The relationship between microbial activity and carbonate precipitation has long been recognized in modern and ancient sedimentary environments, and in laboratory studies (Nadson, 1928; Neher & Rohrer, 1958; Krumbein, 1979; Morita, 1980; Chafetz & Buczynski, 1992; Vasconcelos *et al.*, 1995; Rivadeneyra *et al.*, 1997). Bacteria are common and ubiquitous constituents in modern carbonate sediments and are involved in their formation through metabolic processes, such as photosynthesis, ammonification and sulphate reduction, that increase alkalinity and bicarbonate concentration and induce carbonate precipitation under both oxic and anoxic conditions (Buczynski & Chafetz, 1991; Vasconcelos &

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McKenzie, 1997; Ehrlich, 1998; Visscher *et al.*, 1998; Castanier *et al.*, 1999; Sagemann *et al.*, 1999; Wright, 1999; Warthmann *et al.*, 2000). Encrustment and fossilization of microbes can be studied in recent sediments and may provide information on the processes responsible for mineral formation.

Recent laboratory experiments have shown that microbial sulphate reduction can be responsible for the formation of carbonates with different Mg/Ca ratios (Sagemann *et al.*, 1999; Warthmann *et al.*, 2000). Bacterial sulphate reduction may overcome the kinetic barrier to dolomite formation by increasing pH and carbonate alkalinity (Vasconcelos *et al.*, 1995; Vasconcelos & McKenzie, 1997; Castanier *et al.*, 1999; Wright, 1999; Warthmann *et al.*, 2000) and by removing sulphate, an inhibitor to dolomite formation (Baker & Kastner, 1981). In addition, sulphate occurs in sea water as a magnesium sulphate ion pair, and removal of sulphate ions by bacteria may increase the availability of magnesium ions for dolomite precipitation (Vasconcelos & McKenzie, 1997). Understanding carbonate formation by sulphate-reducing bacteria under anoxic conditions may furnish information on Precambrian carbonate sedimentation, as well as on diagenetic processes that occur in the marine subsurface.

The recognition of microbial carbonates in the geological record is often controversial. Scanning electron microscopy (SEM) of natural or laboratory-produced microbial carbonate has a high potential to reveal the association of bacteria with carbonate minerals (Chafetz & Folk, 1984; Folk, 1994; Rivadeneyra *et al.*, 1998, 2000; Casanova *et al.*, 1999) but does not prove their involvement in mineral formation. In this study, bacterial fossilization processes in recent dolomitic sediment from Lagoa Vermelha, Brazil were investigated. In addition, carbonate precipitates formed in the laboratory by mixed and pure cultures of sulphate-reducing bacteria isolated from Lagoa Vermelha sediments were studied with SEM and compared with the modern environment. To investigate the role of the cell surface of sulphate-reducing bacteria in dolomite nucleation and bacterial fossilization, electrophoretic mobility measurements were carried out to determine the affinity of the cells for magnesium and calcium ions. Insight into the mechanism of microbial fossilization in carbonate sediments may provide clues to recognize microbial carbonate formation and trace it throughout the geological record.

METHOD

Sediment cores were collected from Lagoa Vermelha, a coastal lagoon located about 100 km east of Rio de Janeiro city, Brazil. A microbial dolomite model was first described in Lagoa Vermelha (Vasconcelos & McKenzie, 1997), and sulphate-reducing bacterial cultures from the lagoon have been shown to mediate dolomite precipitation in the laboratory (Warthmann *et al.*, 2000). The upper 1 m of Lagoa Vermelha sediment (up to 98% total carbonate) is characterized by alternating carbonate and organic carbon-rich layers. Low Mg-calcite, aragonite, high Mg-calcite (7–35 mol.% $MgCO_3$) and Ca-dolomite (42–48 mol.% $MgCO_3$) are the carbonate mineral phases present in the lagoon sediment (Höhn *et al.*, 1986; Vasconcelos, 1994; Vasconcelos & McKenzie, 1997). Dolomitic sediment samples from 2 cm depth in the sediment cores were prepared for SEM studies.

A sample of Lagoa Vermelha dolomitic sediment was diluted in a saline phosphate buffer and used to inoculate a Postgate medium (Postgate, 1984). The resulting mixed culture of sulphate-reducing bacteria was grown at 30 °C under anoxic conditions using lactate as a carbon source and sulphate as the sole electron acceptor. Pure bacterial cultures were obtained from the sediment using Lagoa Vermelha medium (Warthmann *et al.*, 2000) for deep agar dilution series. The isolated pure cultures *Desulfovibrio* sp. strains LVform6 and LVform1 (EMBL accession no. AJ548465 and AJ544687 respectively) and a reference strain *Desulfonatronovibrio hydrogenovorans* (Zhilina *et al.*, 1997) were grown in Lagoa Vermelha medium on formate, and incubated without shaking at 30 °C. Parallel sterile control experiments without bacteria were run simultaneously for all experiments. X-ray diffraction spectra of precipitates were obtained using a Scintag diffractometer to identify the minerals present. In order to visualize microbial cells in the samples, the precipitate was treated with DAPI, a highly specific fluorescent stain for DNA. The DNA–DAPI complex fluoresces bright blue when excited with light at a wavelength of 365 nm. Samples were studied using a Zeiss Axioskop-2 phase-contrast microscope with an appropriate filter.

Scanning electron microscope studies were conducted using a Hitachi S-700 field emission SEM. Dolomitic sediment samples from Lagoa Vermelha were chemically fixed in 2·5% glutaraldehyde in 0·2 M Na-cacodylate buffer for 90 min at 4 °C. Cells grown on glass plates and cell mineral pellets from centrifuged cultures

were fixed with 2·5% glutaraldehyde within their culture medium. Fixed samples were subsequently washed with distilled water, 30% ethanol, dehydrated in acetone and critical point dried in liquid CO_2. The samples were coated with 8 nm platinum before SEM imaging. Controls of culture medium without bacteria and minerals were treated as samples and prepared identically for SEM imaging, functioning as background controls. In addition, a methodological comparison was made for some of the samples by cryofixation in 10% methanol, freeze substitution, dehydration in acetone and critical point drying. A comparison of two fixation techniques permitted a better interpretation of the images because fixation artifacts could be excluded.

Sulphate-reducing bacterial strains LVform1, LVform6, *D. hydrogenovorans* and *Desulfovibrio profundus* (Bale *et al.*, 1997) were grown in a marine mineral medium with formate or lactate as a substrate and sulphate as an electron acceptor. Cells from these pure cultures were collected by centrifugation, washed in phosphate-buffered saline and distilled water and resuspended in a small volume of distilled water. The bacterial suspensions were diluted in different electrolytes until the optical density measured at 578 nm was about 0·05 OD, corresponding to about 2500–3000 counts on a Malvern Zetamaster (Malvern Instruments, UK). The electrophoretic mobility of suspended bacteria was measured by dynamic light scattering on the Zetamaster. All samples were measured in duplicate. A Latex standard (GMP, Switzerland) was applied for calibration. In order to determine the isoelectric point, the electrophoretic mobility of the strains was measured at pH 3·5–10·5 in solutions with ionic strengths from 10^{-2} M to 10^{-3} M KNO_3 (Hunter, 1981). Electrophoretic mobility of bacterial cells is an indirect measurement of the cell's surface charge. To study the cation adsorption to bacterial surfaces, the electrophoretic mobility of bacterial suspensions was measured as a function of aqueous H^+, K^+, Na^+, Mg^{2+}, Ca^{2+} and SO_4^{2-} concentrations with ionic strengths from 10^{-4} to 10^{-1} M.

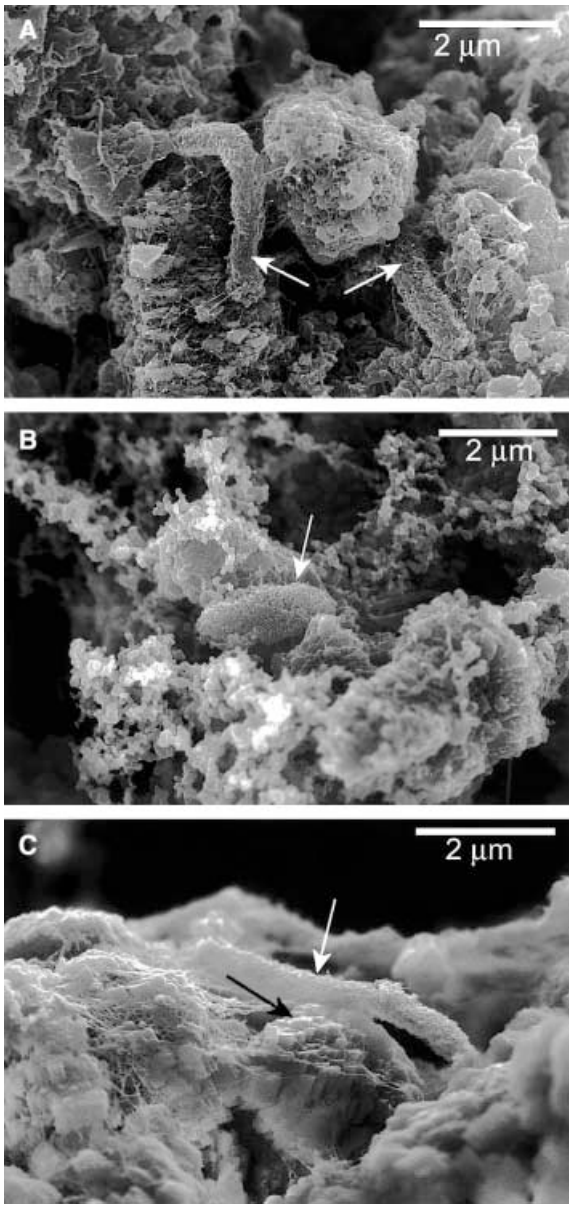

Fig. 1. Scanning electron micrographs of a dolomitic Lagoa Vermelha sediment sample from 2 cm depth. (A) Bacteria (arrow) are closely associated with the sediment particles. The size of the bacteria is similar to that of the isolated pure culture LVform6. (B) Oval-shaped bacterium (arrow) coated with a fine-grained carbonate precipitate. (C) A long, curved, rod-shaped bacterium (arrow) with a fine-grained carbonate precipitate on its surface. The bacterium appears to rest on a layer of dolomite crystals (black arrow) with well-developed orthorhombic shapes.

RESULTS

SEM study of Lagoa Vermelha sediment

Bacteria were found to be closely associated with the carbonate mineral phase in sediment samples taken from 2 cm depth in Lagoa Vermelha (Fig. 1A–C). Some of these bacteria-like bodies had irregular rather than smooth surfaces, indicating that the bacteria are coated by a carbonate precipitate (Fig. 1B and C). Bacteria with both smooth and irregular surfaces were observed, indicating that the latter was not

an artifact of fixation and sample coating for SEM analysis.

Mixed cultures simulating the sedimentary environment

A visible amount of carbonate formed after about 3 weeks in mixed cultures of sulphate-reducing bacteria from Lagoa Vermelha. SEM revealed bacterial colonies consisting of single-type sulphate-reducing bacteria held together by extracellular organic matter. Within bacterial colonies, some bacteria were coated with a fine-grained carbonate precipitate (Fig. 2), demonstrating the process by which colonies become mineralized.

Dolomite formation in pure bacterial cultures

After 3 weeks' incubation of LVform6 and *D. hydrogenovorans*, a visible amount of dolomite formed in the reaction vials. The apparent precipitation rate was $\approx$ 500 mg L^{-1} $month^{-1}$. Dumbbell-shaped bodies consisting of partially ordered, non-stoichiometric dolomite formed in cultures of LVform6 and *D. hydrogenovorans* (Fig. 3A and B respectively; Warthmann *et al.*, 2000). Bacteria were sometimes attached to the dumbbell surfaces, which formed in dense colonies of *D. hydrogenovorans* culture (Fig. 3B). Dolomite nucleated exclusively in bacterial colonies of some 10 000 cells aggregated in an organic matrix composed of extracellular polymeric material (Fig. 3B). Phase-contrast microscopy of a DAPI-stained sample also revealed that the dumbbells were embedded in extracellular material surrounding the biomass (Fig. 4).

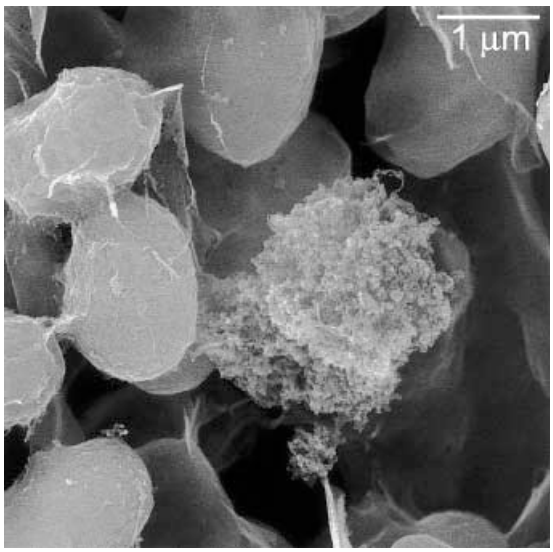

Fig. 2. Scanning electron micrographs of a mixed culture of sulphate-reducing bacteria. Within bacterial colonies, individual bacterial cells are coated with an unidentified fine-grained mineral precipitate. An example of one coated bacterial cell is shown in the centre of the picture.

Electrophoretic mobility measurements

Strain *D. hydrogenovorans* had an isoelectric point at pH 5 with an electrophoretic mobility of $-4{\cdot}6$ m^2 V^{-1} s^{-1}. Strain LVform6 had its isoelectric point at pH 3 with an electrophoretic mobility of $-2{\cdot}5$ m^2 V^{-1} s^{-1}. Electrophoretic mobility measurements of *D. hydrogenovorans* showed two trends: (1) electrolytes with monovalent cations (KNO_3, Na_2SO_4, NaCl) demonstrated a decreasing electrophoretic mobility with increasing ionic strength; and (2) electrolytes with bivalent cations ($CaCl_2$ and $MgCl_2$) demonstrated an increasing electrophoretic mobility with increasing ionic strength, and the charge inverted at high ionic strength (Fig. 5). The electrophoretic mobility of *D. hydrogenovorans*, measured in different electrolytes at 10^{-4} M, varied from -6 to -3 m^2 V^{-1} s^{-1} (Fig. 5).

The sulphate-reducing bacterial strains analysed for their electrophoretic mobility all had different affinities for Mg^{2+}, Ca^{2+}, Na^+ and H^+ (Fig. 6). Relative to the average electrophoretic mobility measured in the most dilute solution, the electrophoretic mobility of bacteria in 0·1 M $MgCl_2$ increased most for strain LVform6, followed by *D. profundus*, *D. hydrogenovorans* and strain LVform1 (Fig. 6). The electrophoretic mobility of bacteria in 0·1 M $CaCl_2$ increased most for *D. hydrogenovorans*, followed by *D. profundus*, strain LVform6 and strain LVform1 (Fig. 6).

DISCUSSION

Bacterial fossilization

Microbes are the most abundant and widespread organisms in sediments and like to adhere to inorganic surfaces (Riding & Awramik, 2000). Therefore, it is no surprise that living bacteria are commonly observed in close spatial relationship with mineral particles (Folk, 1994; Castanier *et al.*, 1999). Fossilized and entombed bacteria, as observed in Lagoa Vermelha sediments (Fig. 1), however, may indicate bacterial involvement in the carbonate precipitation process. Being well aware of potential artifacts, such as fixation, etching and coating artifacts (Kirkland *et al.*,

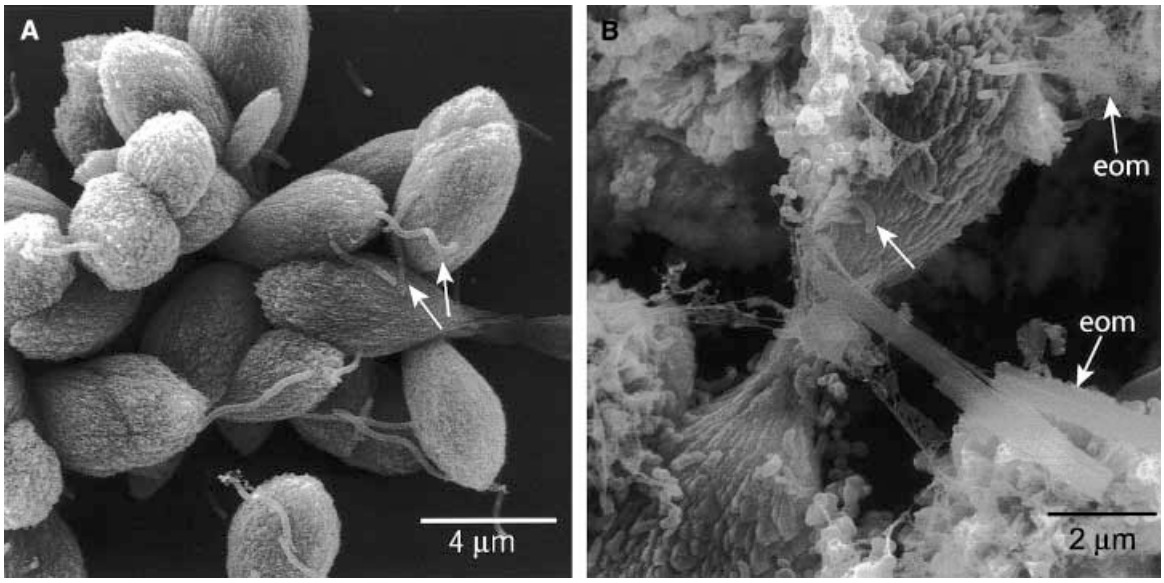

Fig. 3. (A) Sulphate-reducing bacteria of strain LVform6 (arrows) are closely related to the dolomite dumbbells that precipitated in this culture. (B) A dolomite dumbbell, typically embedded in a matrix of sulphate-reducing bacteria *D. hydrogenovorans* (arrow) and extracellular organic material (eom). XRD analysis identified the mineral dumbbells formed in both sulphate-reducing bacterial cultures as nearly stoichiometric and well-ordered dolomite, as also indicated by the well-defined crystal texture visible on the outer surface of the dumbbell.

1999) that may occur during sample preparation for SEM studies, two fixation methods were tested, and a control study was undertaken without bacteria. Chemical fixation and cryofixation led to identical images and the background signal was negligible, indicating that fixation and coating artifacts could be ruled out.

Bacteria were intimately associated with carbonate mineral surfaces in both Lagoa Vermelha sediment samples and bacterial culture experiments. The bacteria-like bodies were observed attached to the mineral surface of Lagoa Vermelha dolomitic sediment (Fig. 1C) or partly entombed (Fig. 1B), in contrast to the integrated bacteria observed by Castanier *et al.* (1999) that emerged perpendicular from crystal planes and eventually left no traces with ongoing precipitation. With etching, Castanier *et al.* (1999) observed rounded structures becoming visible, similar to rounded surface structures on other carbonate mineral surfaces, which have commonly been interpreted as calcified entombed bacterial bodies (Casanova *et al.*, 1999; Castanier *et al.*, 1999; Rivadeneyra *et al.*, 2000). In previous SEM investigations of etched Lagoa Vermelha sediment, entombed bacteria were observed (Vasconcelos *et al.*, 1995; Vasconcelos & McKenzie, 1997), demonstrating that, with etching, more integrated bacteria and bacteria protruding from crystals may become visible. Another reason for not observing deep entombed bacteria may be the low preservation potential of encrusted bacteria. Encrusted bacteria most probably die and the cell lyses, possibly leaving only a cast with the particular shape behind.

The present study of the bacterial mixed culture demonstrates calcification of bacterial clusters, which resembles the well-documented 'biolith' formation process of Rivadeneyra *et al.* (1998). Nucleation and growth of dolomite dumbbells occurred exclusively in an organic matrix in laboratory pure culture experiments, and bacteria (LVform6 and *D. hydrogenovorans*) were found to be associated with the dolomite dumbbell surface. The organic matrix encapsulating the bacteria may promote diffusion gradients, whereby ions can diffuse through and enable the development of the physicochemical conditions promoting dolomite precipitation. Under diffusion-controlled conditions in bacterial colonies, a high concentration of freely available or adsorbed Mg^{2+} would tend to combine with the bicarbonate ions released by the cells; the result would be the nucleation of dolomite microcrystals in close spatial relation with the cells (Warthmann *et al.*, 2000).

Entombed bacteria or bacteria emerging from the crystal planes were not observed in these pure cultures. The close spatial relationship of bacteria and dolomite crystals, however, suggests that bacteria could be entombed by increased dolomite precipitation rates, similar to fossilization processes observed in the sediment. Dolomite

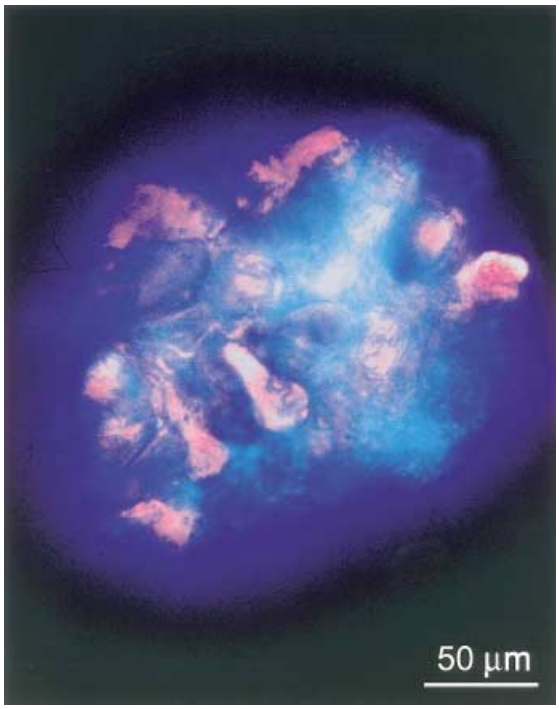

Fig. 4. Dolomite dumbbells embedded in bacterial clusters. This light micrograph shows a bacterial aggregate of $\approx 10^5$ sulphate-reducing bacteria in blue fluorescence by the DNA-binding DAPI dye. The purple to bright blue colour represents the concentration of cells. The dolomite minerals also show some fluorescence, but at a different wavelength (bright pink), not representing the DNA–DAPI complex. Dolomite dumbbells in this culture were formed exclusively in bacterial aggregates.

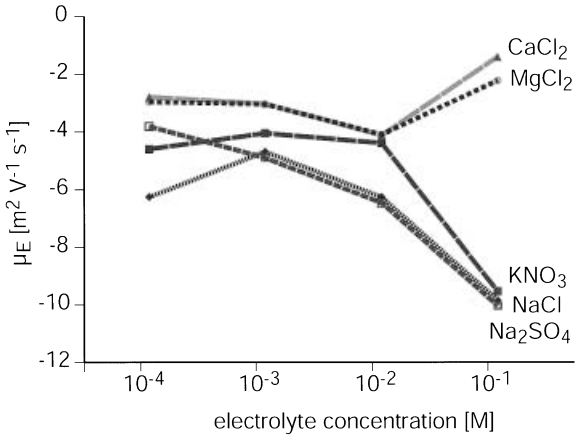

Fig. 5. Electrophoretic mobility (μE) of *D. hydrogenovorans* measured in electrolyte $CaCl_2$, $MgCl_2$, KNO_3, NaCl and Na_2SO_4 with ionic strengths ranging from 10^{-4} to 10^{-1} M.

formed in the bacterial pure culture experiments at a rate of ≈ 500 mg L^{-1} $month^{-1}$. This rate was obtained in experiments beginning with low bacterial density and 40 mM formate and 10 mM sulphate. In Lagoa Vermelha, where the bacterial density is higher and the sulphate concentration is greater at 50 mM, the carbonate crystallization rate is faster. Differences in carbonate precipitation rate may also explain the different fossilization of mixed and pure bacterial cultures. The mixed cultures may simulate bacterial fossilization in the natural environment better because of higher carbonate precipitation rates than the pure cultures.

Involvement of the cell surface

The isoelectric point of strain *D. hydrogenovorans* and LVform6 was determined at low pH, respectively 0·5 to 2·5 pH units lower than the isoelectric point reported for other sulphate-reducing bacterial strains (Ulanovskii *et al.*, 1980). The indication that the isoelectric point was at low pH for these strains demonstrates that the bacterial surface consists of proteins, lipopolysaccharides and peptides with carboxyl, hydroxyl, amino and phosphate groups, slightly dominated by the acidic groups. This implies that, at a neutral pH (pH > pH isoelectric point), the bacterial surface is negatively charged. The different trends in electrophoretic mobility, measured in monovalent and bivalent electrolytes with increasing ionic strength, indicate that monovalent cations interacted with the cell surface differently than bivalent cations.

Previous research on the electrophoretic mobility of bacterial cells has shown that cations affected the electrostatics at the negatively charged bacterial surfaces, but anions did not (Simoni *et al.*, 2000). The negative charge of bacterial surfaces is counterbalanced by positive charges in the diffuse ion cloud in the electrolyte. This causes a decrease in electrophoretic mobility with increasing ion strength, as observed in electrolytes comprising monovalent cations (Fig. 6). Bivalent cations bind specifically to phosphate and carboxyl surface groups and reduce the net charge within the electrokinetic shear plane. This implies that fewer counterions accumulate in the diffusive layer (Hunter, 1981). Specific binding of bivalent cations explains the initial decrease in electrophoretic mobility with increasing ionic strength and the eventual inversion of charge (Fig. 6). Bivalent cations are known to stabilize the outer membrane of Gram-negative bacteria by reducing the charge repulsion between highly anionic lipopolysaccharide molecules (Coughlin *et al.*, 1983). The increased

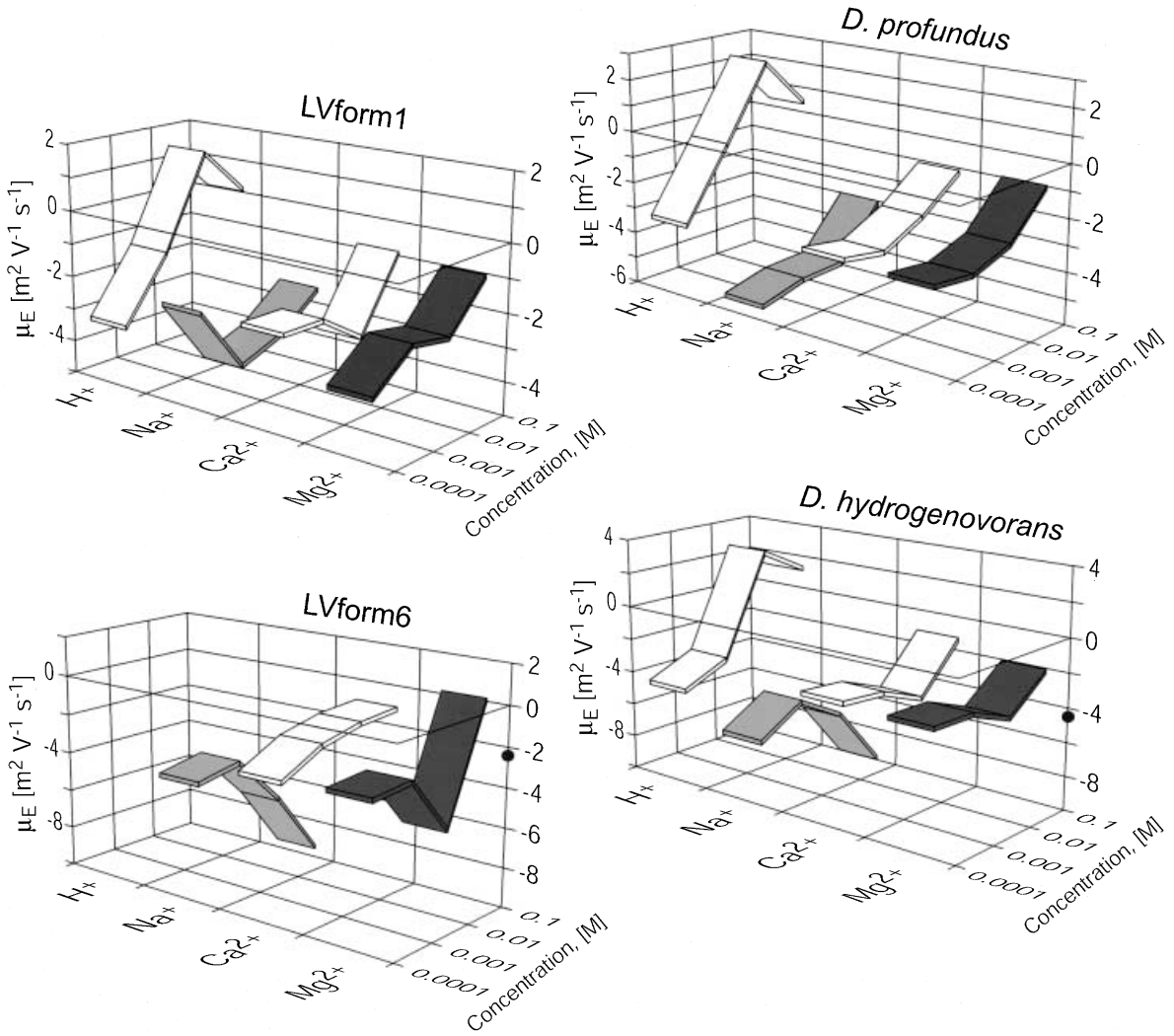

Fig. 6. Electrophoretic mobility (μE) of strains LVform1, LVform6, *D. profundus* and *D. hydrogenovorans* measured in HCl, NaCl, $CaCl_2$ and $MgCl_2$ with ionic strengths ranging from 10^{-4} to 10^{-1} M, reflecting the adsorption behaviour of cations to the cell surface. The black circles on the vertical axis represent the point of zero charge of the respective bacterial cells.

electrophoretic mobility with increased electrolyte concentration indicates that both Mg^{2+} and Ca^{2+} ions were specifically adsorbed to the bacterial surface of strain LVform6, LVform1, *D. hydrogenovorans* and *D. profundus*. This is consistent with previous research showing that Mg^{2+} and Ca^{2+} are the main ions bound to the outer membrane of Gram-negative bacteria (Coughlin *et al.*, 1983).

The electrophoretic mobility at the point of zero charge was similar to the average electrophoretic mobility measured in most dilute electrolytes. A comparison demonstrates that *D. hydrogenovorans* has the largest negative permanent surface charge, followed by *D. profundus*, strain LVform6 and LVform1. All analysed sulphate reducers specifically adsorbed Mg^{2+} and Ca^{2+} cations; Mg^{2+} ions were adsorbed most strongly by strain LVform6 and *D. hydrogenovorans*, strains that were also found to mediate dolomite formation. Sulphate-reducing bacteria take up sulphate without Mg^{2+} (Warthmann & Cypionka, 1990), thereby dissociating the $[MgSO_4]^0$ ion pair and making Mg^{2+} available to adsorb to the cell surface. Mg^{2+} and Ca^{2+} are concentrated at the cell surface, where bicarbonate is also produced. This leads to a supersaturation of the microenvironment around the cell promoting dolomite nucleation. Ca^{2+} ions, adsorbed onto bacterial surfaces, can induce the precipitation of fine-grained calcium carbonate by the complexation of counterions (Schultze-Lam *et al.*, 1996; Fortin *et al.*, 1997; Douglas & Beveridge, 1998). In the same way, it is proposed here that specific

binding of Mg^{2+} and Ca^{2+} ions on the bacterial surface may catalyse dolomite formation by the complexation of carbonate counterions.

CONCLUSIONS

This study shows that an intimate association of sulphate-reducing bacteria and carbonate minerals exists in both Lagoa Vermelha sediments and bacterial culture experiments. It is proposed that carbonate precipitation was mediated by the bacteria in both cases. In the Lagoa Vermelha sediments, the occurrence of encrusted, calcified bacterial bodies provides evidence of the sulphate-reducing bacterial mediation of carbonate formation, as also demonstrated in laboratory culture experiments. In bacterial mixed cultures, bacterial clusters were found to be mineralized as a whole, whereas in pure cultures, the bacterial cells were associated only with the surfaces of carbonate minerals, and could be entombed with continuing carbonate precipitation. Fossilized bacteria are an indicator of microbial carbonates in the sedimentary rock record.

Ca^{2+} and Mg^{2+} were found specifically to adsorb onto the negatively charged surface of halotolerant, alkaliphilic sulphate-reducing bacteria. In addition to the sulphate-reducing activity, the bacterial cell surface of sulphate-reducing bacteria is involved in the mediation of dolomite precipitation through the capability of concentrating Mg^{2+} around the cells where dolomite nucleation occurs. Bacterial fossilization is thus a result of the microbial mediation of carbonate precipitation.

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