Exopolymeric substances of sulfate-reducing bacteria: Interactions with calcium at alkaline pH and implication for formation of carbonate minerals

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ABSTRACT

Sulfate-reducing bacteria (SRB) have been recognized as key players in the precipitation of calcium carbonate in lithifying microbial communities. These bacteria increase the alkalinity by reducing sulfate ions, and consuming organic acids. SRB also produce copious amounts of exopolymeric substances (EPS). All of these processes influence the morphology and mineralogy of the carbonate minerals. Interactions of EPS with metals, calcium in particular, are believed to be the main processes through which the extracellular matrix controls the precipitation of the carbonate minerals. SRB exopolymers were purified from lithifying mat and type cultures, and their potential role in CaCO$_3$ precipitation was determined from acid-base titrations and calcium-binding experiments. Major EPS characteristics were established using infrared spectroscopy and gas chromatography to characterize the chemical functional groups and the sugar monomers composition. Our results demonstrate that all of the three SRB strains tested were able to produce large amounts of EPS. This EPS exhibited three main buffering capacities, which correspond to carboxylic acids ($pK_a = 3.0$), sulfur-containing groups (thiols, sulfonic and sulfinic acids – $pK_a = 7.0–7.1$) and amino groups ($pK_a = 8.4–9.2$). The calcium-binding capacity of these exopolymers in solution at pH 9.0 ranged from 0.12 g Ca g$^{-1}$ EPS$^{-1}$ to 0.15 g Ca g$^{-1}$ EPS$^{-1}$. These results suggest that SRB could play a critical role in the formation of CaCO$_3$ in lithifying microbial mats. The unusually high sulfur content, which has not been reported for EPS before, indicates a possible strong interaction with iron. In addition to changing the saturation index through metabolic activity, our results imply that SRB affect the rock record through EPS production and its effect on the CaCO$_3$ precipitation. Furthermore, EPS produced by SRB may account for the incorporation of metals (e.g. Sr, Fe, Mg) associated with carbonate minerals in the rock record.

INTRODUCTION

Seawater is supersaturated with respect to calcium carbonate, but precipitation does not occur spontaneously due to various inhibiting factors. These factors include the high hydration energies of Ca$^{2+}$ and Mg$^{2+}$ (Slaughter & Hill, 1991), ion-pairing with sulfate, and presence of organic ligands that binds Ca$^{2+}$ and Mg$^{2+}$ (Wright & Oren, 2005). In lithifying microbial mats (i.e. where calcium carbonate precipitates are found), the metabolic activity of sulfate-reducing bacteria (SRB), which are abundant in mats (Canfield & DesMarais, 1991; Fründ & Cohen, 1992; Visscher et al., 1992), is believed to enhance calcium carbonate precipitation (Visscher et al., 2000; Dupraz et al., 2004; Baumgartner et al., 2006). The activity of SRB affects the formation of carbonate minerals in several ways. Firstly, sulfate reduction results in a pH increase, affecting the saturation index and thus directly the precipitation of carbonate minerals (Lyons et al., 1984; Walter et al., 1993; Visscher & Stolz, 2005). Secondly, when SRB use low molecular weight organic acids (e.g. lactate, acetate) as electron donors for growth, the availability of free calcium ions may increase due to the removal of carboxylic acids binding calcium (Bosak, 2005; Dupraz & Visscher, 2005). Thirdly, by removing sulfate ions from solution, SRB alter the kinetic
inhibition of dolomite formation (Wright, 1999; Warthmann et al., 2000; van Lith et al., 2003a,b; Wright & Wacey, 2005). Through these processes, the metabolic activity of SRB can be considered as the environmental ‘engine’ that sustains carbonate precipitation in lithifying microbial mats. In addition, the mere presence of SRB cells, even metabolically inactive, may favour calcium carbonate precipitation by providing heterogeneous nucleation sites (Ferris, 2000; Bosak & Newman, 2003).

In a laboratory investigation, Desulfovibrio sp., isolated from a corroded surface, chelated metals such as iron, chromium, nickel and molybdenum through production of exopolymeric substances (EPS) (Beech & Cheung, 1995; Zinkevich et al., 1996; Beech et al., 1999). Other studies showed that cyanobacterial EPS was able to bind metal ions such as cadmium, manganese, copper, lead, mercury and calcium to various functional groups that were present in sugars and amino acids constituting the EPS (Somers & Brown, 1978; Mohamed, 2001; Mehta & Gaur, 2007). The negatively charged surface of the cyanobacterial EPS-containing sheath was thought to favour nucleation and precipitation of calcium carbonate (Pentecost, 1985; Riding, 2000). The presence of cyanobacterial EPS also increased the viscosity of the medium, acting as a diffusion barrier, impacting calcium ions mobility, kinetics of precipitation, and consequently the mineralogy of calcium carbonate (Buczynski & Chafetz, 1991). The direct role of cyanobacteria on the CaCO$_3$ polymorph that precipitated was demonstrated using EPS from Schizothrix sp. (Kawaguchi & Decho, 2002). A similar role in carbonate geochemistry could be ascribed to the EPS produced by aerobic heterotrophic bacteria (Hardikar & Matijevic, 2001; Braissant & Verrecchia, 2002; Kim et al., 2005; Lian et al., 2006), and, by extension, probably other groups of bacteria present in biofilms and microbialites (Dupraz & Visscher, 2005).

In lithifying microbial mats, the precipitation of carbonate minerals takes place in an EPS-rich matrix (Dupraz et al., 2004), and remnants of this EPS may be preserved in the rock record (Barbieri & Cavalazzi, 2005; Altermann et al., 2006; Benzerara et al., 2006). The EPS characteristics control the type and quantity of the calcium carbonate minerals produced (Kawaguchi & Decho, 2002; Braissant et al., 2003). Degradation of EPS-containing sheath material was associated with dolomite formation (Wright & Altermann, 2004). The interactions between the metal ions and the EPS are mediated by several functional groups (i.e. specific groups of atoms such as carboxylic acids or amino groups) present in the EPS matrix. Deprotonation of functional groups takes place when the pH increases, providing a negative charge to the polymer (Somers & Brown, 1978; Cox et al., 1999; Socrates, 2001; Phoenix et al., 2002; Fig. 1). In addition to sugar monomers, the EPS matrix may include noncarbohydrate acidic moieties such as pyruvate, succinate, and functional groups such as sulfate or phosphate (Sutherland, 2001a,b,c,d), which also contribute to the overall negative charge of the EPS.

The geochemical role of SRB metabolism in the precipitation of CaCO$_3$ has been well documented in lithifying mats (Visscher et al., 1992; Reid et al., 2000). Previous work has shown that uncharacterized EPS produced by Desulfovibrio desulfuricans G20, isolated from an oil well corrosion site, altered the CaCO$_3$ mineral morphology (Bosak & Newman, 2005). This important geomicrobiological attribute of SRB has not been assessed in cultures obtained from microbial
mats. Here, we present data that supports the Bosak & Newman (2005) hypothesis that EPS produced by SRB influence the mechanisms of calcium carbonate precipitation. We tested three SRB strains, including isolates from a modern marine stromatolite and a lithifying hypersaline mat, for EPS production. The acid-base properties and the calcium-binding capacity of this EPS were determined to investigate the effects of functional groups on mechanisms of calcium carbonate precipitation.

**MATERIALS AND METHODS**

**Bacteria and culture conditions**

Three strains of EPS-producing sulfate reducers were used in this study. Strains belonging to the genus *Desulfobacterium* were isolated from a stromatolite surface (Highborne Cay, Bahamas; (76°51′W; 24°42′N); strain: H0407_12.1La; GenBank accession number: DQ822785) and from a microbial mat (Salt Pan, Eleuthera, Bahamas; (76°34′W; 25°24′N); strain LM1), respectively (Baumgartner et al., 2006). The third strain was *Desulfo bacterium autotrophicum* (Bak & Widdel, 1986; Brysch et al., 1987), a well-known marine type strain, kindly provided by Dr Leadbetter.

The bacteria were cultured in modified Bak & Widdel (1986) medium, consisting of: KH₂PO₄ 0.2 g L⁻¹, NH₄Cl 0.25 g L⁻¹, KCl 0.5 g L⁻¹, CaCl₂·2H₂O 0.15 g L⁻¹, MgCl₂·6H₂O 3.0 g L⁻¹, NaCl 20 g L⁻¹ (except for strain LM1, which was grown with 60 g NaCl L⁻¹), and 10 mm Na₂SO₄. After autoclaving and cooling to 60 °C, the following solutions were added: selenite-tungstate solution 1 mL L⁻¹ (NaOH 0.5 g L⁻¹, Na₂SeO₃·5H₂O 6 mg L⁻¹, Na₂WO₄·2H₂O 8 mg L⁻¹), mixed vitamins solution 1 mL L⁻¹ (4-aminobenzoic acid 40 mg L⁻¹, D(+)-biotin 10 mg L⁻¹, nicotinic acid 100 mg L⁻¹, Ca-D-(+)-panthotenate 50 mg L⁻¹, pyridoxine·2HCl 150 mg L⁻¹, thiamine·2HCl 100 mg L⁻¹), vitamin B₁₂ solution 1 mL L⁻¹ (cyanocobalamin 50 mg L⁻¹), SL10 trace element solution (FeSO₄·7H₂O 2.1 mg L⁻¹, MnCl₂·4H₂O 100 mg L⁻¹, CoCl₂·6H₂O 190 mg L⁻¹, ZnSO₄·7H₂O 144 mg L⁻¹, H₂BO₃ 6 mg L⁻¹, NiCl₂·6H₂O 24 mg L⁻¹, CuCl₂·2H₂O 2 mg L⁻¹, NaMoO₄·2H₂O 36 mg L⁻¹, HCl 37% 8 mL L⁻¹), and 0.1% resazurin solution 0.5 mL L⁻¹. Finally, the medium was cooled under N₂/CO₂ (80%/20%) flow and 30 mL L⁻¹ of a 1 M NaHCO₃ solution (30 mM final concentration), 8 mL of a 0.2 M Na₂S solution (1.6 mM final concentration) to reduce the medium, and 20 mL of a 1 M Na-lactate solution (20 mM final concentration) were added.

Growth of cultures was monitored by measuring the optical density at 600 nm spectrophotometrically (using a Varian Model Cary 50; Varian Inc., Palo Alto, CA, USA). The protein content of the cells was measured using the bicinchoninic acid assay (BCA, Sigma, St. Louis, MO, USA; Smith et al., 1985) with bovine serum albumin (BSA) as standard. The carbohydrate content of the culture was estimated using the phenol-sulfuric acid assay (Dubois et al., 1956). The EPS content was assayed using Alcian Blue 8GX (Acros Organics, Geel, Belgium) (Passow & Alldredge, 1995; Bober et al., 2005). In this assay, 6 mL of cold ethanol was added to 2 mL of culture sample to precipitate EPS. The sample was then centrifuged for 20 min and the supernatant was discarded. The tube containing the EPS pellet was allowed to air dry for 1 h and after which 2 mL of 0.15 mg mL⁻¹ Alcian Blue 8GX in 5% acetic acid was added. The mixture was allowed to react for 1 h, after which the absorbance was read at 614 nm. Xanthan was used as a standard. In addition to this assay, EPS dry weight was measured at the end of the culture growth.

**EPS extraction and purification**

Exopolymers substances of SRB were recovered in the early stationary growth phase. Under these conditions, acid-base and calcium-binding properties of the EPS were shown to be consistent in replicate experiments. The culture was prefiltered twice through a glass fibre filter (Whatman GF/F, Whatman Inc., Florham Park, NJ, USA) followed by a single filtration through a 0.2 μm nitrocellulose filter (Sartorius Corp., Edgewood, NY, USA). The EPS in the filtrate was precipitated by adding cold ethanol (4 °C) in a 1:1 ratio. Precipitation was allowed to take place for at least 3 h at 4 °C. The EPS was recovered by centrifugation, placed in dialysis tubing (10–12 kDa) and dialysed against de-ionized water (>18 MΩ). After dialysis, the EPS was stored at 4 °C or freeze dried.

**Acid-base titration**

The acid-base titration was used to determine the proton-binding sites and gain insight into potential types and densities of the functional groups present in the EPS. For the acid base titration, 2–3 mL of the dialysed EPS (approximately 6.5 mg of dry EPS) was dissolved in de-ionized water (>18 MΩ) to obtain a final volume of 40 mL. The solution was transferred to an anaerobic chamber (COY laboratory product, Grass Lake, MI, USA) under nitrogen (98%) – hydrogen (2%) atmosphere to avoid the formation of carbonate ions due to dissolution of atmospheric CO₂. The initial pH of the solution was adjusted to 3.0, which typically required between 100 μL and 150 μL of 1 N HCl, and the solution was titrated with 0.1 N NaOH added stepwise in 10 μL increments. The pH was recorded until pH 11 was reached using an Orion 720 A pH meter (Orion, Boston MA, USA). All reagents were prepared with autoclaved de-ionized water that was allowed to cool to room temperature under vacuum. The titration curves obtained were analysed using the PROTOFIT 2.1 software (Turner & Fein, 2006).

**Calcium titration**

The calcium chloride titration was carried out to establish the maximum calcium-binding capacity of the SRB exopolymers. This titration was carried out according to Shimomura &
Inouye (1996) under nitrogen (98%) – hydrogen (2%) atmosphere to avoid the formation of carbonate ions due to dissolution of atmospheric CO₂. EPS was dissolved in a solution containing 40 mM KCl and 20 mM Tris-OH, which was adjusted to pH 9.0. The titration was carried out by adding a CaCl₂ solution (0.1 or 1 M) in increments of 10 μL to 40 μL to the EPS solution. The concentration of free calcium ions was recorded with a calcium ion-selective electrode (Cole-Palmer Instrument Co., Vernon Hills, IL, USA) coupled to a high-impedance millivolt meter (Microscale Measurement, The Haag, Netherlands). All reagents were prepared using autoclaved de-ionized water that was allowed to cool to room temperature under vacuum. The EPS used for measurement, The Haag, Netherland). All reagents were prepared using autoclaved de-ionized water that was allowed to cool to room temperature under vacuum. The EPS used for measurement, The Haag, Netherland). 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Sulfate-reducing bacteria exopolymeric substances

Fig. 2 Growth of the sulfate-reducing bacteria Desulfovibrio sp. (H0407_12.1Lac), expressed by optical density and protein production. Note the correlation between the protein content and the exopolymer content ($r^2 = 0.92$), suggesting that exopolymers are produced continuously.

Table 1 Protein, exopolymeric substances (EPS), and the ratio of protein to EPS, produced in the cultures of the different sulfate-reducing bacteria. Cells and EPS were harvested when the early stationary growth phase was reached. EPS was precipitated, freeze-dried and weighed.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Protein [mg L$^{-1}$]</th>
<th>EPS [mg L$^{-1}$]</th>
<th>Protein/EPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desulfobacterium autotrophicum</td>
<td>574 ± 18</td>
<td>239 ± 13</td>
<td>2.4</td>
</tr>
<tr>
<td>Desulfovibrio sp.*</td>
<td>743 ± 2</td>
<td>239 ± 10</td>
<td>3.1</td>
</tr>
<tr>
<td>Desulfovibrio sp.†</td>
<td>235 ± 21</td>
<td>164 ± 6</td>
<td>1.4</td>
</tr>
</tbody>
</table>

*Strain H0407_12.1Lac isolated from a stromatolite mat.
†Strain LM1 isolated from a hypersaline lithifying microbial mat.

Table 2 Analysis of the monomeric sugar composition in the carbohydrate fraction of extracellular polymeric substances (EPS) from different strains of sulfate-reducing bacteria. Values are expressed in mole percentage. Rha, rhamnose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose.

<table>
<thead>
<tr>
<th></th>
<th>Desulfobacterium autotrophicum</th>
<th>Desulfovibrio sp.*</th>
<th>Desulfovibrio sp.†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rha</td>
<td>n.d.</td>
<td>46.2</td>
<td>n.d.</td>
</tr>
<tr>
<td>Xyl</td>
<td>28.0</td>
<td>7.2</td>
<td>10.6</td>
</tr>
<tr>
<td>Man</td>
<td>15.2</td>
<td>16.2</td>
<td>14.0</td>
</tr>
<tr>
<td>Gal</td>
<td>9.0</td>
<td>1.3</td>
<td>19.3</td>
</tr>
<tr>
<td>Glc</td>
<td>47.8</td>
<td>29.1</td>
<td>56.1</td>
</tr>
</tbody>
</table>

*Strain H0407_12.1Lac isolated from a stromatolite mat.
†Strain LM1 isolated from a lithifying microbial mat.

Table 3 Apparent pK values observed in the acid-base titration of exopolymeric substances isolated from SRB cultures during early stationary phase.

<table>
<thead>
<tr>
<th>Strain</th>
<th>pK$_1$</th>
<th>pK$_2$</th>
<th>pK$_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desulfobacterium autotrophicum</td>
<td>3.2*</td>
<td>7.0</td>
<td>9.2</td>
</tr>
<tr>
<td>Desulfovibrio sp.*</td>
<td>3.0*</td>
<td>7.0</td>
<td>8.7</td>
</tr>
<tr>
<td>Desulfovibrio sp.†</td>
<td>3.0*</td>
<td>6.9</td>
<td>8.5</td>
</tr>
</tbody>
</table>

*Strain H0407_12.1Lac isolated from a stromatolite mat.
†Strain LM1 isolated from a lithifying microbial mat.
*Lower pK values are expected below the range of the present titration.

Fig. 3 (A) Acid-base titration of the exopolymers produced by Desulfobacterium autotrophicum showing three buffering zones (dots). The first zone located around pH 3.2 and below is attributed to carboxyl groups. The second zone located at pH 7.0 is attributed to thiol groups. The third buffering zone at 9.2 is attributed to amino groups. Note that the carboxyl-buffering region can be determined by comparison with the control titration (dashed line). Control titration has been performed using the same volume of deionized water adjusted to the same initial pH with HCl 1 N. (B) Derivative of the acid-base titration curve showing the equivalence points and the apparent pKs values for this exopolymer (dots). Peaks indicate a maximum variation in pH corresponding to the equivalence points and local minima indicate a minimum variation in pH, which is indicative of buffering. Local minima may not appear at the beginning and end of the titration curve due to the small variation in pH. Arrows indicate the corresponding pH value of the titration curve for each equivalence points (Eq.) and half-titration points (i.e. pKs – pKa$_n$). Solid line represents a bicubic interpolation used to smooth the data.

derivative of the titration curve (Fig. 3B), comparison with the control revealed a clear difference between the two curves (Fig. 3A). This particular area of buffering was expected to extend beyond pH 3.0, as typical pK values for carboxylic acids range from 1 to 5 (Stumm & Morgan, 1996). The second buffering zone was located at pH 7.0. In view of the large amount of sulfur reported in the EPS of SRB (Beech & Cheung, 1995), this buffering could be attributed to thiol, sulfinic or sulfonic acid groups (Fig. 3). FT-IR spectral data (see below) supported this interpretation, and many thiols, sulfinic and sulfonic acid groups of bioorganic molecules have a pK close to 7.0 (Danehy & Noel, 1960; Kreinov et al., 1960; Stumm & Morgan, 1996). The final buffering zone appeared between pH 8.5 and 9.2. Usually this buffering capacity has been attributed to amino groups (Phoenix et al.,
In view of the observed sulfur content of the SRB-EPS in our study, it could be possible that both thiol and amino groups contributed to this third buffering zone. Thiols have a wide range of pKa values (Stumm & Morgan, 1996) including at alkaline pH. Moreover, amino and thiol groups may participate in buffering at higher pH values than investigated here. Similar to our observation for the carboxyl groups, buffering from amino groups was not evident from the derivative of the titration curve, but can be visualized when compared with the titration curve of the control.

Using the PROTOFIT 2.1 software (Turner & Fein, 2006), and assuming three binding sites, the pKa of the proton-binding sites as well as their densities could be estimated (Table 4). Using this software, the first pKa was found between 2.5 and 2.6. There is a slight discrepancy between the fitted values and those observed from the titration curve. A lower pKa range in the fitted data implies that a greater buffering capacity could be expected at low pH values than revealed by the titration. The lower estimated buffering could be attributed to carboxylic acids or to sulfate groups that were present in large amounts in the EPS. Typical pKa values for sulfate groups are usually below 2.5 (Schiewer, 1999). The second fitted buffering zone, between 6.9 and 7.0, corresponding to sulfur compounds (e.g. thiols, sulfinic or sulfonic acids) matched the observed data. The third buffering zone, corresponding to amino groups was estimated by the software between 8.8 and 9.4, is close to the value observed from the titration curve directly. For the three strains, the estimated binding site densities for carboxylic acids, sulfur compounds, and amino groups were estimated to 2.4–2.5 mol kg\textsuperscript{-1}, 1.5–1.7 mol kg\textsuperscript{-1} and 1.5–1.7 mol kg\textsuperscript{-1}, respectively. These observations emphasize the importance of functional groups other than carboxylic acid in the geochemical properties of EPS.

### Functional group

<table>
<thead>
<tr>
<th>Strain</th>
<th>pK\textsubscript{1}/SD\textsubscript{pK1}</th>
<th>pK\textsubscript{2}/SD\textsubscript{pK2}</th>
<th>pK\textsubscript{3}/SD\textsubscript{pK3}</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Desulfobacterium autotrophicum</em></td>
<td>2.51 ± 0.08/2.45 ± 0.05</td>
<td>7.03 ± 0.04/1.52 ± 0.01</td>
<td>9.41 ± 0.04/1.52 ± 0.03</td>
</tr>
<tr>
<td><em>Desulfovibrio</em> sp.*</td>
<td>2.62 ± 0.03/2.41 ± 0.08</td>
<td>6.99 ± 0.01/1.70 ± 0.05</td>
<td>8.95 ± 0.01/1.66 ± 0.02</td>
</tr>
<tr>
<td><em>Desulfovibrio</em> sp.†</td>
<td>2.55 ± 0.08/2.46 ± 0.12</td>
<td>6.98 ± 0.01/1.71 ± 0.02</td>
<td>8.80 ± 0.05/1.75 ± 0.03</td>
</tr>
</tbody>
</table>

*Strain H0407_12.1Lac isolated from a stromatolite.
†Strain LM1 isolated from a lithifying microbial mat.

**Table 4** Exopolymeric substances (EPS) pKa values calculated from the EPS acid-base titration curve using PROTOFIT (Turner & Fein, 2006), assuming three binding sites

**Fourier-transform infrared (FT-IR) spectroscopy of EPS**

FT-IR analyses (Fig. 4) showed that EPS from *Desulfovibrio* strain H0407_12.1Lac contained several major infrared absorption peaks. Absorption peaks at 1033 and 1057 cm\textsuperscript{-1} were assigned to carbohydrate C-O stretching vibrations. A peak at 1109 cm\textsuperscript{-1} could be attributed to S = O, stretching vibration from sulfinic or sulfonic acid (Coates, 2000; Socrates, 2001). The peak at 1650 cm\textsuperscript{-1} was likely due to C = C stretching vibrations, which exist in a vinylidene (i.e. 2H on the same C) conformation (890 cm\textsuperscript{-1}). A doublet at 2340 and 2350 cm\textsuperscript{-1} could be attributed to the O-H stretching vibrations from sulfonic or sulfonic acids (Coates, 2000; Socrates, 2001). Peaks at 2920 cm\textsuperscript{-1} represented C-H stretches, and 3340 cm\textsuperscript{-1} represented O-H stretches. Substantial amounts of sulfate were present in EPS derived from SRB isolates, as determined by covalent sulfate peaks at 1161 cm\textsuperscript{-1}, representing an S-O stretch accompanied by a deformation
at 610 cm\(^{-1}\), and a peak at 1316 cm\(^{-1}\). Although their sugar monomer composition was different (Table 2), the FT-IR spectra for the two other strains (not shown) displayed identical peaks. The sulfate content was estimated at 15–18%, when quantified using dextran sulfate standards. The results of FT-IR analyses also suggested that carboxyl groups could have been present in smaller amounts in these EPS polymers, as they typically form a prominent peak near 1630–1650 cm\(^{-1}\), with a shoulder at 1730 cm\(^{-1}\). Proteins were not detected in the purified EPS by the BCA assay. Therefore, it is unlikely that these peaks could be attributed to amides associated with proteins. Finally, an absorption peak located at 898 cm\(^{-1}\) could be attributed to the \(\beta\)-glycoside linkage between sugar monomers. The major FT-IR peaks in the natural EPS sample were nearly identical to those observed in the pure cultures.

**EPS calcium titration**

The titration with calcium chloride revealed that calcium was bound to EPS when compared to the control. At low concentrations of calcium, most was bound or formed complexes and, hence, was not measured by the ion-selective electrode. As the concentration of calcium increased, the available EPS calcium-binding sites were increasingly saturated until no additional calcium was bound. This clearly occurred, as demonstrated by the plateau when plotting bound calcium against calcium added (Fig. 5). The calcium-binding capacity of the EPS was determined using the fitted curve (Fig. 5). Under our experimental conditions, these maximum calcium-binding capacities for *D. autotrophicum* and the *Desulfovibrio* strain H0407_12.1Lac isolated from a microbial mat were 0.15 g\(\text{Ca}\) g\(\text{EPS}^{-1}\) and 0.12 g\(\text{Ca}\) g\(\text{EPS}^{-1}\), respectively.

**DISCUSSION**

The data presented here constitute, to our knowledge, the first report on the acid-base chemical properties and metal (i.e. Ca\(^{2+}\))-binding capacity for EPS produced by sulfate-reducing bacteria. While the titration data describe the overall chemical properties of the exopolymeric substances, FT-IR determines the functional groups, which are responsible for the observed specific chemical properties. Many functional groups have similar pKs, and titration only is insufficient for characterizing EPS. This is relevant because of the pivotal role of SRB in many environmental and industrial processes such as biomineralsation and corrosion, metal binding, bioremediation and biofouling (Hines *et al*., 2002; Megonigal *et al*., 2003).

The results from the acid-base and calcium titrations show that SRB-EPS have a strong potential to exchange protons and calcium ions with the surrounding (micro)-environment. Moreover, the amount of EPS produced in cultures of the three strains tested shows that this extracellular component cannot be neglected. Extreme daily fluctuations of the geochemical gradients, especially of pH, are characteristic for natural mats (Visscher *et al*., 1998; Dupraz *et al*., 2004). Hence, the different chemical response of the various functional groups constituting the EPS is noteworthy. Carboxylic acid and sulfate groups will always remain deprotonated as pH in the microbial mat will not decrease below pH values of 4.0. This allows the carboxylic acid and the sulfate groups to be efficient metal-binding sites under any typically encountered pH in the lithifying mats. On the contrary, thiol-, sulfinic, sulfonic acid and amino groups with pK values ranging from 7.0 to 9.0 will be subject to periodic changes in protonation states. In other words, these functional groups will not always be able to bind metals, as typical pH in microbial mats may vary from 6.5 to 10.5 (Visscher *et al*., 1998). However, the functional groups with high pK (i.e. pH > 7.0) values may act as an ‘environmental buffer’ by initially releasing protons when the pH increases, and similarly, binding protons when the pH decreases. This assists in creating an optimal physiological pH for the SRB and other microbes, and could explain the role of the abundant sulfur groups in the SRB exopolymer. A change in pH from 6.5 to 10.5 will affect the surface charge...
density of the polymer (Fig. 6). In the organomineralization model proposed by Trichet & Défarge (1995), a geometrical change in surface charge induces a template for calcium binding and CaCO₃ nucleation.

The values for calcium binding in this study appear to be high. It is likely that the affinity for calcium is lower in the natural environment due to variations in pH and the presence of competing divalent cations such as magnesium, iron, barium and strontium. Laboratory experiments with xanthan as model EPS show a decrease of the affinity for calcium in the presence of magnesium or strontium (Braissant, 2005). Similarly, data from Disnar & Trichet (1984) demonstrate that copper, cobalt and nickel compete for binding sites in natural exopolymers. However, the binding capacities reported for cadmium (up to 0.43 gCd g⁻¹ EPS; Mohamed, 2001) and manganese (up to 0.91 gMn g⁻¹ EPS; Mohamed, 2001; Freire-Nordi et al., 2005; Mehta & Gaur, 2007) to cyanobacterial EPS suggest that our Ca-binding values are realistic.

In modern stromatolites and lithifying microbial mats, sulfate-reducing bacteria are closely associated with calcifying aragonitic micritic layers and high-Mg calcite micritic layers (Visscher et al., 2000; Dupraz et al., 2004). The acid-base and Ca-binding properties of SRB-EPS described here will influence the SI of carbonate minerals as well as their morphology and mineralogy. Three main processes characteristic of EPS matrices have been shown to control the precipitation of carbonate minerals: (1) if calcium concentration exceeds the EPS-binding capacity under suitable pH conditions (i.e. pH > 8.4, which value represents the stability of carbonate minerals), precipitation will occur inside the EPS matrix due to local super saturation (Arp et al., 2003); (2) self (re-)arrangement of acidic functional groups in the EPS matrix may create a template that favours the nucleation of carbonate minerals. This process is often referred to as organomineralization (Trichet & Défarge, 1995); and (3) degradation of EPS by heterotrophic bacteria will contribute to the release of calcium, increasing the SI. Microbial EPS degradation promotes carbonate precipitation by replacing the decaying polymers by (high-Mg) calcite (Dupraz et al., 2004; Decho et al., 2005; Visscher & Stolz, 2005). Many heterotrophic bacteria are known to degrade and grow on various types of EPS (Nankai et al., 1999; Sutherland, 1995, 1999; Hashimoto et al., 1998; Visscher et al., 1999). In this context, release of Ca bound to SRB-EPS is likely to enhance carbonate minerals precipitation. The involvement of SRB-EPS is also supported by the remarkable similarity of FT-IR spectra of EPS extracted from stromatolite mats (Reid et al., 2000) and SRB-EPS (Fig. 5). Such resemblance of EPS from cultures and natural samples suggests that SRB are major contributors to the natural EPS pool, in the stromatolite lithifying layers where high SRB activity is observed. Other organisms such as cyanobacteria also produce large amounts of polymers containing sulfur functional groups and may have a similar influence. Clearly, this requires further investigation.

The high sulfur content of the EPS in our study indicates that both volatile and nonvolatile sulfur-containing degradation products may be produced during decomposition. Interestingly, high levels of sulfonates were found in a number of microbial mats (Visscher et al., 1999), which were thought to represent an important energy and carbon source for anaerobic heterotrophs (Lovley & Coates, 2000). In addition, the presence and flux of a number of volatile sulfur compounds have been measured in a variety of microbial mats (Visscher & van Gemerden, 1991; Visscher et al., 1994, 2003; Taylor & Visscher 1996; Jonkers et al., 1998), which could potentially result from EPS degradation. The source of the large amounts of volatile compounds typically emitted from mats that greatly impacted the Earth’s atmosphere (Pilcher, 2003), has been tied to SRB metabolism (Visscher et al., 2003).

Finally, the present study focused on the importance of EPS–calcium interactions. However, in the geological record, calcium carbonate minerals often include traces of other metal cations such as magnesium, iron and strontium. The interaction of EPS with these metals may lead to the formation of ankerite (Ca(Fe,Mg)(CO₃)₂) and dolomite (CaMg(CO₃)₂), which are common in sedimentary rocks. In addition, previous X-ray photoelectron spectroscopy studies have shown that SRB-EPS has a strong affinity for iron (Beech et al., 1999). The results from the present study suggest that this affinity for iron may be mediated by sulfur groups such as thiols, sulfenic acids or sulfonic acids, all of which have a high affinity for this metal (Ferris, 2000).

CONCLUSION

This study demonstrated that sulfate-reducing bacteria isolated from lithifying microbial mats were able to produce large amounts of EPS. Investigation of this EPS revealed a buffering capacity, which was sustained by sulfates, carboxylic acids, thiols, sulfenic acids and amino groups. In addition, the
present study showed that this SRB-EPS has a strong calcium-binding capacity. We conclude that, in addition to their role in increasing alkalinity in modern stromatolites and lithifying microbial mats, SRB have the ability to interact with calcium. They can promote or inhibit the formation of carbonate minerals but also create templates enhancing different mineralogies. SRB played an important role during the geochemical evolution of Earth (Canfield et al., 2000; Paytan, 2000). Therefore, the SRB mechanisms that mediate carbonate mineral formation are of great interest for the interpretation of early rock record. The present work documents the role of the SRB’s sulfur-rich EPS matrix, and suggests a role of these polymers in the formation of biosignatures, especially (sulfur-rich) kerogen compounds associated with carbonates (Sinninghe Damste et al., 1989).

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REFERENCES


Sulfate-reducing bacteria exopolymeric substances


