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Bacterial Activity and Preservation of Sedimentary Organic Matter: The Role of Exopolymeric Substances

Muriel Pacton
Department of Geology-Paleontology, University of Geneva, Geneva, Switzerland

Nicolas Fiet
UMR-CNRS 8148, Bat. 504, Paris-Sud University, 91405 Orsay Cedex, France

Georges Edouard Gorin
Department of Geology-Paleontology, University of Geneva, Geneva, Switzerland

Although exopolymeric substances (EPS) are associated with the microorganisms contributing to the production/degradation of sedimentary organic matter, their role in theses processes have so far never been mentioned. Using high-resolution microscopical tools (scanning and transmission electron microscopy, atomic force microscopy), fossil organic matter in the Miocene Monterey Formation (California) and Kimmeridgian laminites (France) has been compared with its present-day analogs, i.e., respectively sulphuroxidizing bacteria and cyanobacterial biofilms. This comparison shows that, particularly in the case of Kimmeridgian cyanobacterial mats deposited in a shallow back-reef environment, organic matter preservation is conditioned by exopolymeric substances secreted by bacteria. A model is proposed for the evolution through time of exopolymeric substances in relation to the mechanical constrains they have been exposed to, during lithification and diagenesis. This model is based on the microscopical observation of sulphuroxidizing bacteria and could explain the morphology of fossil organic matter usually referred to as "amorphous" in standard light microscopy. The highly hydrated nature of exopolymeric substances helps to protect organic matter from degradation and remineralization. These substances can be observed only in microscopy and are undetectable through organic geochemical methods, hence the need to combine these two methods in organic matter studies. Consequently, exopolymeric substances must be considered as an important contributing agent to organic matter preservation. These results confirm the complexity of the bacterial role in geoenvironments and add a new parameter in the productivity-vs-preservation debate.

Keywords amorphous organic matter, cyanobacterial biofilm, microscopy, Miocene black shales, Kimmeridgian bituminous shales, sulphuroxidizing bacteria

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Address correspondence to Muriel Pacton, Department of Geology-Paleontology, University of Geneva, 13 rue des Maraîchers, 1205 Geneva. Switzerland. E-mail: Muriel.Pacton@terre.unige.ch

INTRODUCTION

The preservation of sedimentary organic matter (OM), leading to the formation of oil source rocks and oil shales, results from different mechanisms directly related to the environment of deposition. Four main mechanisms of OM preservation are recognized in the literature: recondensation, selective preservation, vulcanization and adsorption. The degradation/recondensation (or recondensation) pathway is characterized by the absence of source organism morphological features. This model is based on a diagenetic fragmentation of the OM biomacromolecules. Monomers are transformed by re-organization into a group of humic substances which, as a whole, further evolve into geopolymers as a result of polycondensation (Tissot and Welte 1984). The selective preservation pathway is based on the production by some living organisms, especially microalgae, of insoluble biomacromolecules highly resistant to chemical and microbial degradation. These biomacromolecules are not affected by strong basic and acid hydrolyses and also show a high resistance to microbial attacks (Goth et al. 1988; Tegelaar et al. 1989; Largeau et al. 1990). The natural vulcanization is an intermolecular incorporation of inorganic sulphur species into low-molecular-weight functionalized lipids resulting in the formation of high-molecular-weight sedimentary OM (Sinninghe Damsté et al. 1988, 1989; Kohnen et al. 1991; Adam et al. 1993). The sorptive protection pathway emphasizes the protective role of minerals. For example, within clays, the alternation of OM and clay nanolayers suggests a physical protection mechanism (Salmon et al. 2000).

These processes are related to the nature of OM constituents, the redox conditions and organomineral relationships. Nevertheless, none of these mechanisms takes into account an important component of bacterial activity, the exopolymeric substances (EPS), which are a dominant constituent of biofilms.

This research, positioned at the interface between geology and biology, focuses on the EPS in order to better understand the contribution of bacteria in the formation of fossil OM. The aims

of this paper are to illustrate the role of EPS in OM preservation and its behavior throughout diagenesis. EPS have been studied in fossil OM, especially in amorphous organic matter (AOM), and compared to those of recent bacteria.

EXOPOLYMERIC SUBSTANCES (EPS) IN THE RECENT AND FOSSIL RECORD

Most bacteria in their natural environments produce extracellular polymeric substances (EPS), which are accumulated outside cells to form a protective and adhesive matrix that attaches them to substrates (Costerton et al. 1978, 1995). Biofilm structures consist of aggregated microbial communities embedded within the EPS, (for a review, see Sutherland 2001). The biofilm community is heterogeneous in nature and is composed of various microorganisms within a chemically variable EPS matrix (Lawrence et al. 1999).

Biofilm EPS are mainly composed of polysaccharides, protein and nucleic acids (Lazarova et Manem 1995; Grady et al. 1999; Lawrence et al. 2003) separated by a network of open water channels (Stoodley et al. 2002). Moreover, extracellular DNA might have a structural role in biofilm formation and stability (Whitchurch et al. 2002; Steinberg and Holden 2005). The biofilm consists of a highly hydrated polyanionic matrix (>90% water) surrounding the bacterial cell and can be composed of hundreds to thousands of monomeric EPS units (Erlandsen et al. 2004).

EPS matrix provides physical and chemical protection. It can also contribute to nutrient absorption (Christensen and Characklis 1990), facilitate metabolic interaction, and act as a diffusion barrier and absorbent (Lawrence et al. 1994). EPS serve as nutrient (Wolfaardt et al. 1995), water (Roberson and Firestone 1992), retention and protection from toxins such as antibiotics (Stewart 1996, 1998) and pollutants (Wolfaardt et al. 1994). Significant active biomass fractions are observed at all depths in biofilms (Hu et al. 2005).

EPS exist in a variety of forms and have recently been subdivided into three subtypes: linear, branched and cyclic (Starkey et al. 2004). On sediment surfaces, they range from well-defined protective envelopes surrounding cells, as in cyanobacterial sheaths, to masses that anchor and provide a favourable medium for microbial populations and communities. In addition, microbes can form blooms (dense concentrations of phytoplanktic cells) and floccules, as in marine and lake snow (millimetric EPS-based organic aggregates suspended in the water column) (Riding 2000).

Recent EPS appears in cryo-scanning electron microscopy (SEM) as large masses of dense filaments segregated from the protein network, whereas in conventional SEM it can be observed as thin filaments randomly mixed with a protein network. It exhibits a well-defined porous network and a denser, entangled appearance, and contains randomly distributed and relatively thicker filaments (Hassan et al. 2003). The formation of a 3-dimensional EPS network is an indication of a gel-type structure (De Vuyst and Degeest 1999). The degree of ropiness (i.e., the property of being cohesive and sticky) in fermented milk is thought to be determined by the physicochemical EPS characteristics (Ruas-Madiedo et al. 2002), because no direct relation has been observed between ropiness and the amount of EPS produced. The degradation of EPS has been demonstrated by Decho et al. (2005) in the case of heterotrophic degradation. Specifically, the sulphate-reducing bacteria transform rapidly EPS to a more refractory remnant polymer.

Although numerous geochemical evidences testify of the presence of microbial activity in fossil OM (biomarkers, $^{13}C_{org}$), microscopy is the most definite technique to identify it. Lithified bacterial biofilms associated with fossilized soft tissues of animal and vegetal macrofossils are well known in the fossil record (Martill 1987; Franzen 1994; Martill and Wilby 1994; Dunn et al. 1997; Liebig 1998). A study of fossils in the Eocene Messel Formation (Germany) revealed that the preservation of some soft tissues is due to their replacement by bacterial biofilms (Wuttke 1983). After death, organic constituents of bacterial cells undergo diagenetic processes resulting in the production of bacterial biomarkers such as bacteriohopanes (Peter and Moldowan 1993). Typical features exhibiting a smooth, sometimes fibrillar or ropy texture in Oligocene deposits resemble those of a marine biofilm (Toporski et al. 2002). Another example is given by Arp et al. (1998) from microbialites in alkaline salt lakes, where detrital particles such as quartz and feldspar grains, allochthonous carbonate crystals, partly calcified pellets, and arthropod remains, are bound within the biofilm.

Early shrinkage of the EPS results in a spatial accumulation of seed crystals forming layers or patches separated from the accumulated detrital components. The filamentous mat is subsequently transformed into an organic mucus within which structures are poorly defined. The mucus substances serve as a Ca^{2+} buffer, preventing mineral precipitation at first, an effect that protects microorganisms and their microenvironments. Nucleation and precipitation does not start until the buffer capacity is overcome. Polysaccharides are generally very hydrophilic because of their polar OH-groups. Finally, in the Kimmeridgian bituminous shales at Orbagnoux the presence of bacterial sheaths has been revealed through sedimentological characteristics (Tribovillard et al. 1999; Pacton et al. 2006).

METHODS

Fossil OM has been geologically sampled in the black shales of the Miocene Monterey Formation (Isaacs 2001) at Naples Beach in California (Figure 1) and in the Kimmeridgian bituminous shales (Bernier 1984; Tribovillard et al. 1999; Pacton et al. 2006) at Orbagnoux in the French Jura (Figure 2). The position of the rock samples is indicated in the lithological columns shown in Figure 3. Recent bacteria used as analogs are both sulphur oxidizing bacteria (taken from a refrigerated sample in Switzerland) and a cyanobacterial biofilm grown in a laboratory culture from a microbial mat sampled in Lagoa Vermehla (Brazil; Vasconcelos 1994).

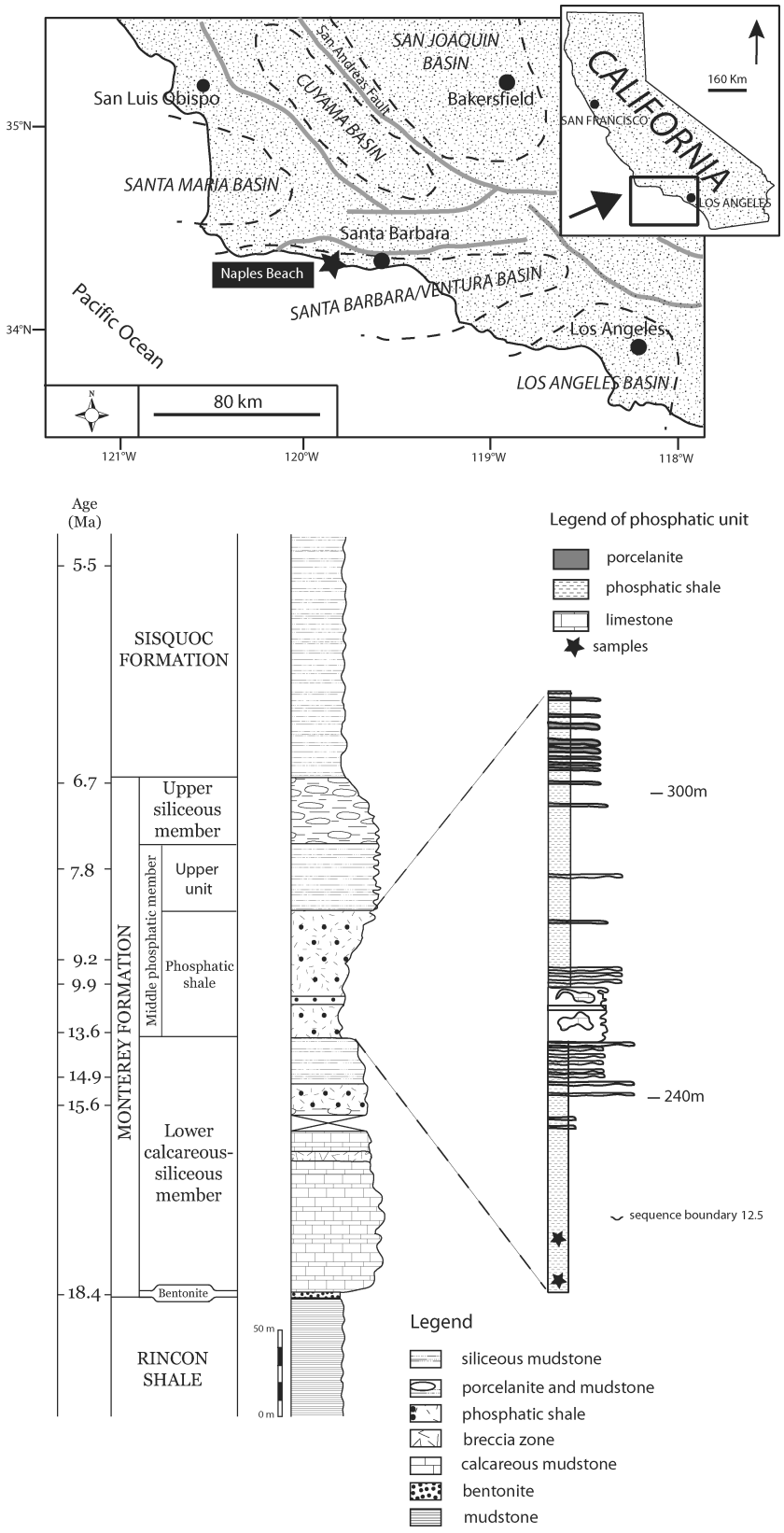

FIG. 1. Location of the Naples Beach section in the Miocene Monterey Formation. Stratigraphic sequences of the Monterey Formation at Naples Beach (modified after Isaacs 2001).

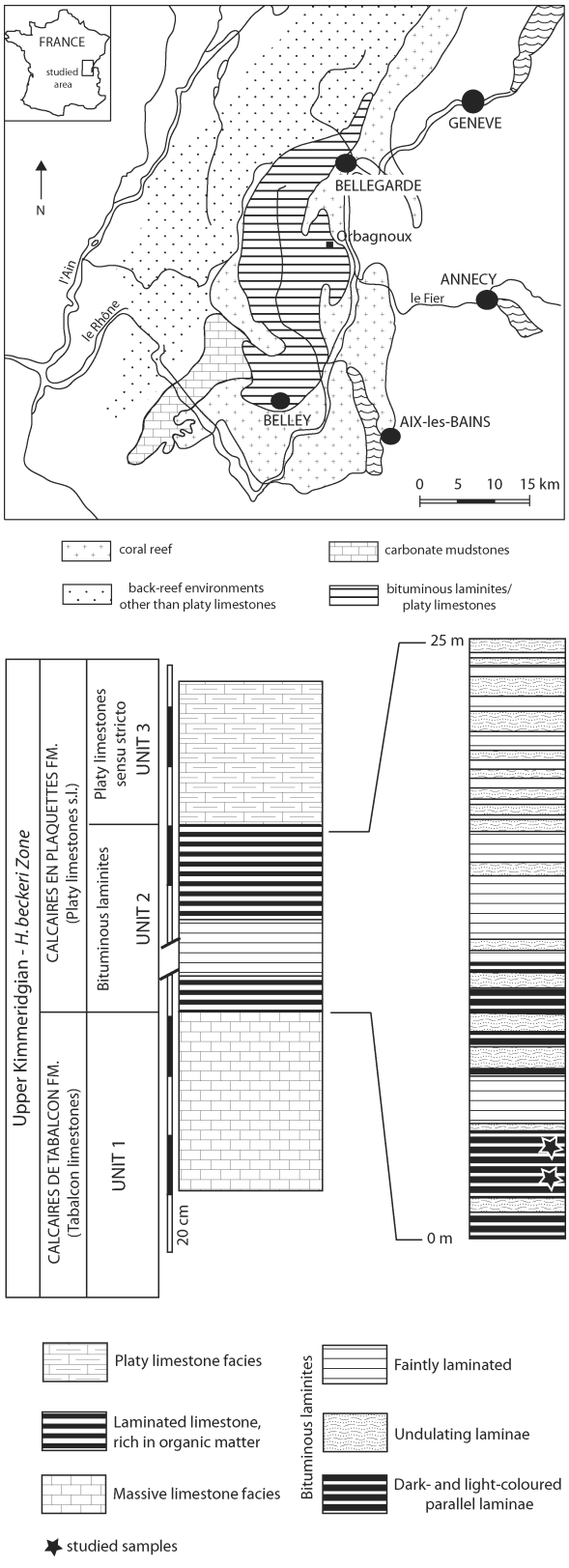

FIG. 2. Palaeogeography of the Upper Jurassic in the southern Jura (modified after Bernier, 1984) and schematic lithological column of the bituminous laminites at Orbagnoux (modified after Tribovillard et al. 2000).

Observation of fossil OM in consolidated rocks requires a standard palynological preparation technique in order to isolate OM from the mineral fraction. This technique includes HCl and HF acid treatment followed by heavy liquid separation (Steffen and Gorin 1993). The studied, present-day sulphur oxidizing bacteria and cyanobacterial biofilm, have been exposed to the same acid treatment for a proper comparison with fossil OM. Subsequently, various microscopical techniques have been used to analyze the OM morphology and structure down to the nanoscale: SEM (JEOL JSM 6400) on gold-coated samples, transmission electron microscopy (TEM) on ultrathin sections and atomic force microscopy (AFM).

In SEM and AFM, EPS in recent bacteria were dried using the critical point drying method, because of their highly hydrated structure. For TEM observations, bacteria were fixed in 1% glutaraldehyde in 50 mM cacodylate buffer at pH 7.4 (CB) and at room temperature for 1 hour, washed 3 times in CB and postfixed in 1% osmium tetroxyde in CB for one hour at room temperature. After 3 washes in CB, the cells were embedded in 2.5% agarose and dehydrated before inclusion in Epon. Thin sections (70 nm) were obtained using a Leica ultramicrotome and contrasted with uranyl acetate.

TEM was performed using a Phillips CM100 transmission electron microscope, and digital image processing was applied. The atomic force microscope (AFM) maps surface topography. In phase imaging, a variant of tapping mode, the phase lag of the canteliver oscillation relative to the signal sent to the cantilever's piezo driver, is used as a basis for image generation. AFM observations were conducted using a Digital Instruments (Veeco) Nanoscope III Dimension 3100 housed at the Laboratory of Geology (Paris-Sud University), at room temperature and air. The probe consisted of a cantilever with integrated silicon nitride tip (Digital instruments). Micron-scale images were acquired using tapping mode. The mechanical behavior of EPS was studied in both recent samples. Prior to acid etching, it is possible to observe in SEM the behavior of EPS in relation with the bacterial density after the fixation procedure described here.

RESULTS

EPS Morphology

Prior to acid etching, EPS in sulphur oxidizing bacteria are characterized in SEM by a succession of veils (Figure 3a) or agglomerated filaments (Figure 3b). This spatial repartition is also visible in TEM where structures are marked by an alveolar network without defined geometric forms (Figure 3c). Alveoli have a diameter of about 40 nm. After acid treatment, part of the EPS network is destroyed, whereas that bound to mineral particles is preserved. Moreover, the dimensions of alveoli are relatively amplified through this treatment (100 nm, Figure 3d).

Prior to acid etching, EPS in the cyanobacterial biofilm do not seem to be constituted of recognizable individual layers but rather than of a dense mass where different kinds of bacteria

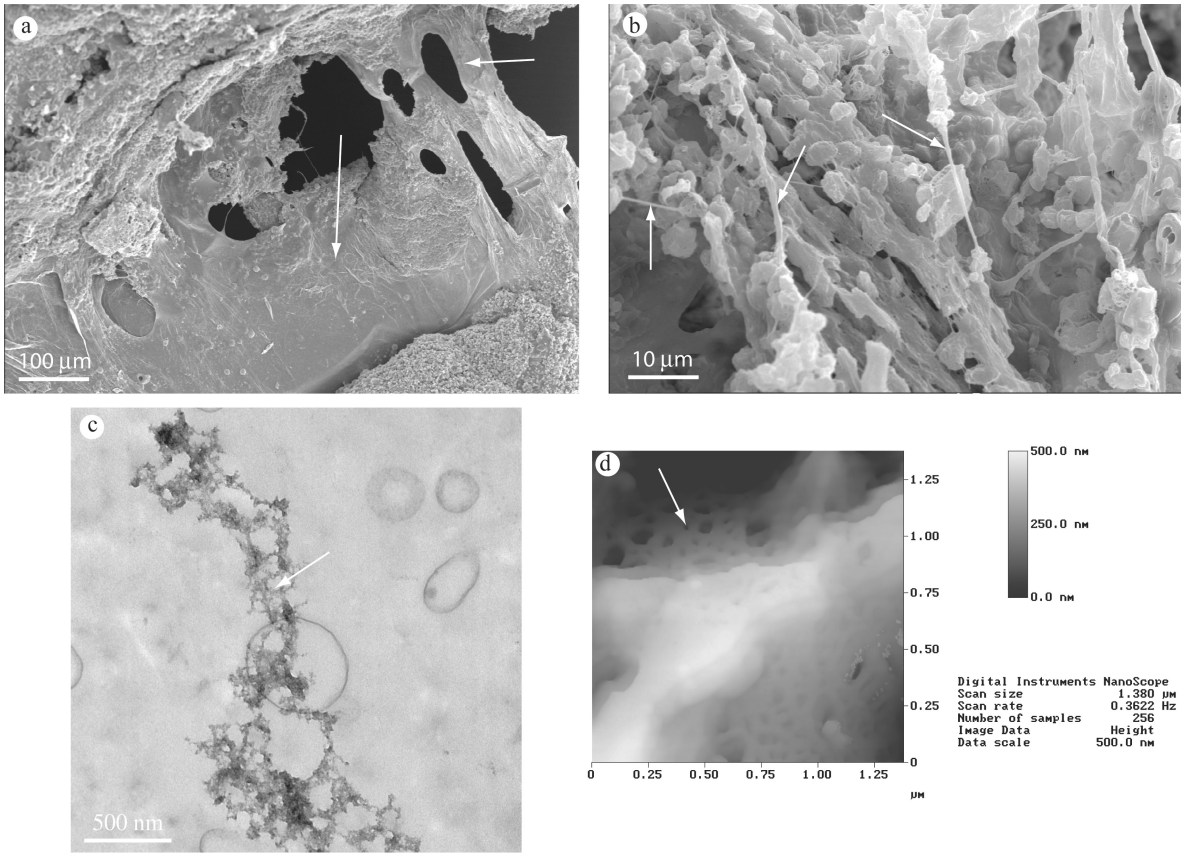

FIG. 3. Recent EPS in sulphur-oxidizing bacteria, *prior to acid etching*: (a) veils formed by EPS (SEM photograph); (b) filaments of agglomerated EPS (SEM photograph); (c) EPS alveolar network in TEM, ultrathin section.*After acid etching*: (d) EPS alveolar network (AFM photograph).

bound to each other are visible, especially filaments (Figure 4a). Locally, a secondary network (probably the last generation of EPS) expands at surface with dendrite-shaped ramifications (Figure 4b). When bacteria are embedded in EPS and not overlying them, the EPS surface displays very weak variations in relief and appears smooth (height variations of less than 3 nm; Figure 4c). However, when the bacterial population is more abundant, the surface aspect is marked by an alveolar network, the diameter of alveoli varying from 25 to 100 nm (Figure 4d). After acid etching, EPS are more condensed and characterized by an alveolar network with different orders of magnitude, from μm to $\sim$10 nm (Figures 5a and 5b).

Mechanical Behavior of Recent EPS

In the case of sulphuroxidizing bacteria, when there are no bacterial constraints, EPS appear as a continuous veil (Figure 6a). When bacteria are locally present, the veil tears off under the action of stretching constraints (Figure 6b). The more important the constraints, the larger the tears, thereby forming an alveolar network (Figure 6c). At a later stage, alveoli merge into filaments terminated by bacteria and a small-size alveolar network stands out around the dense bacterial mass (Figure 6d). However, when successive layers of EPS are stacked together, the biofilm seems to be more resistant and, although several filaments are isolated, the structure generally presents a continuous aspect.

In the cyanobacterial biofilm, EPS of different chemical nature are secreted by each type of bacteria. EPS may form a dense mass (Figure 7a), which is resistant to constraints created by bacteria. On top of the biofilm, the last EPS generation is not anymore bound to the compact mass and forms a dendritic network (Figure 7b). When EPS are submitted to high constraints, e.g., through colonies of diatoms, they display an alveolar network (Figure 7c). In this case, EPS and bacteria form a solid groundmass and cannot be dissociated.

EPS in Fossil OM

In the fossil AOM of the Miocene Monterey Formation, for which sulphuroxidizing bacteria are thought to be a present-day analogue, EPS present different aspects. AOM is locally characterized by alveolar network whith recognizable filaments (Figure 8a). It appears both as an agglomerated mass possibly due to compaction and as a secondary EPS generation with a dendritic form (Figure 8b). In TEM, it shows a more compact and fluffy aspect than in recent OM, but the general geometry is preserved (Figure 8c and 8d).

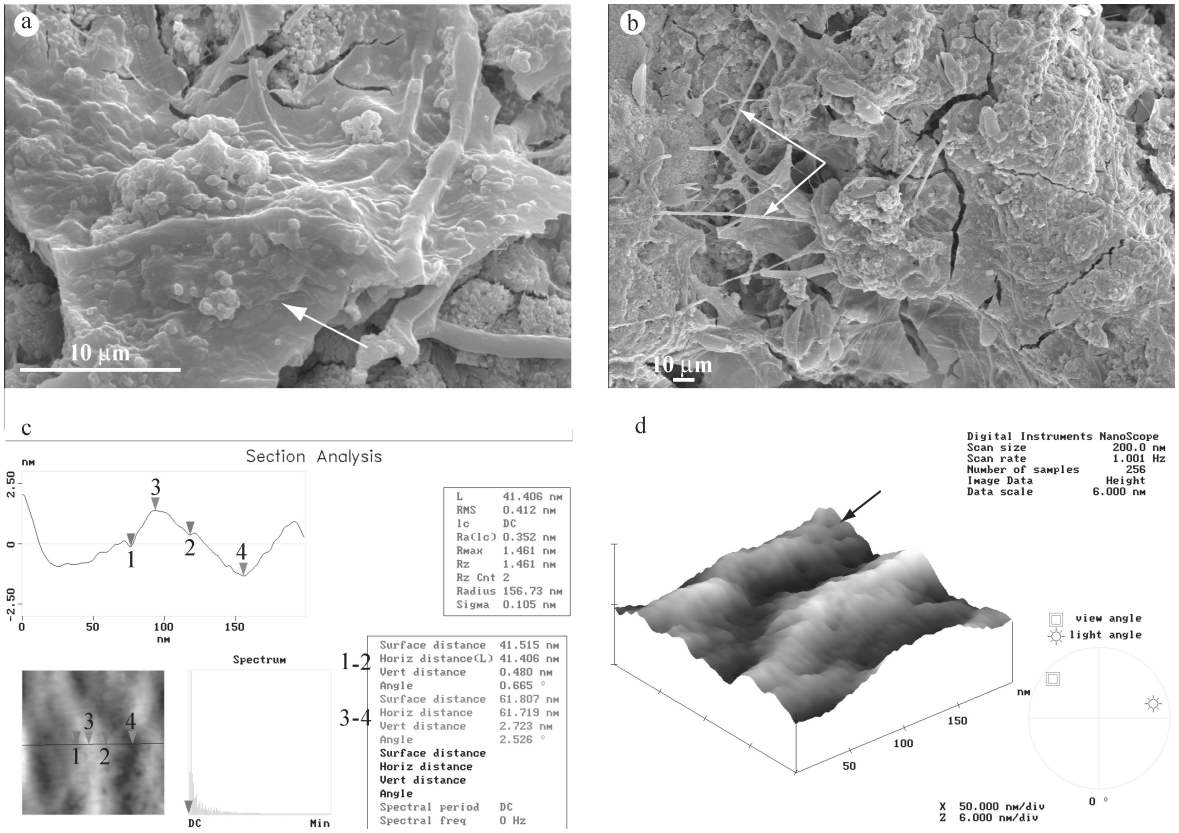

FIG. 4. Recent EPS in a cyanobacterial biofilm, *prior to acid etching*: (a) compact mass of EPS (SEM photograph); (b) EPS as dendroid filaments (SEM photograph); (c) smooth area in EPS without bacteria (AFM photograph); (d) alveolar network in EPS (AFM photograph).

In the Kimmeridgian bituminous shales at Orbagnoux, AOM is mainly characterized by an alveolar microstructure with different orders of magnitude (from several μm to 100 nm, Figure 9a). The general geometry resembles that of a cyanobacterial biofilm characterized by EPS network inbetween bacterial filaments (Figures 9b and 9c). In both cases, the smaller size of structures in fossil samples is probably due to diagenetic processes such as dehydration and compaction.

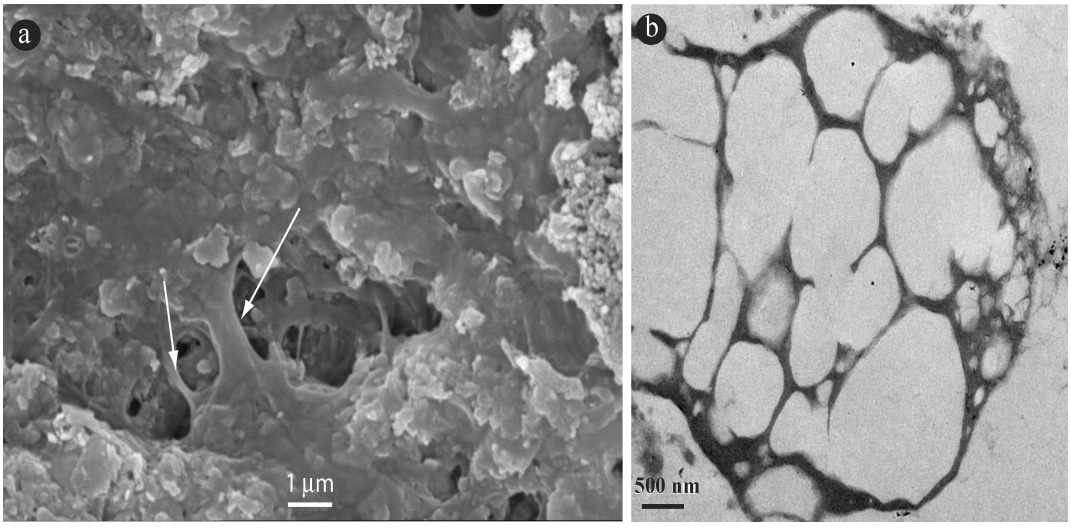

FIG. 5. Recent EPS in a cyanobacterial biofilm *after acid etching*: (a) alveolar network in SEM; (b) alveolar network in TEM, ultrathin section.

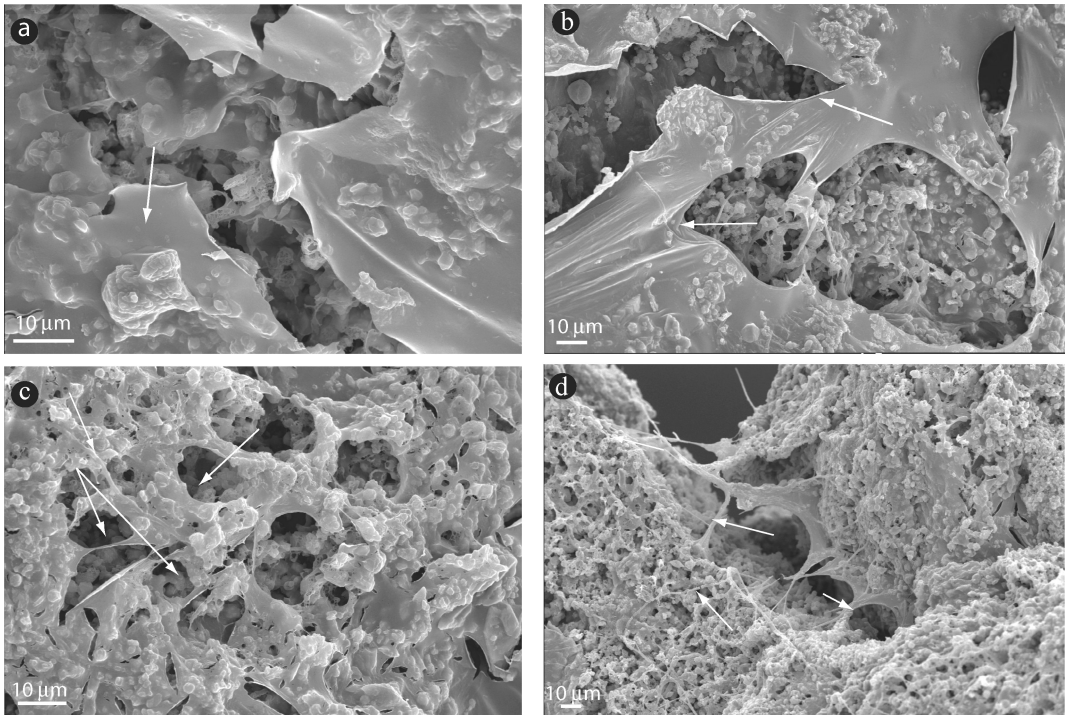

FIG. 6. Mechanical behavior of recent EPS in sulphur-oxidizing bacteria *without acid etching* (SEM pictures): (a) continuous EPS veil in the absence of bacteria; (b) torn off EPS veil resulting from bacterial stretching constraints; (c) alveolar network caused by increasing mechanical constraints; (d) at a later stage EPS compacts into filaments and a small-size alveolar network surrounds a dense bacterial mass.

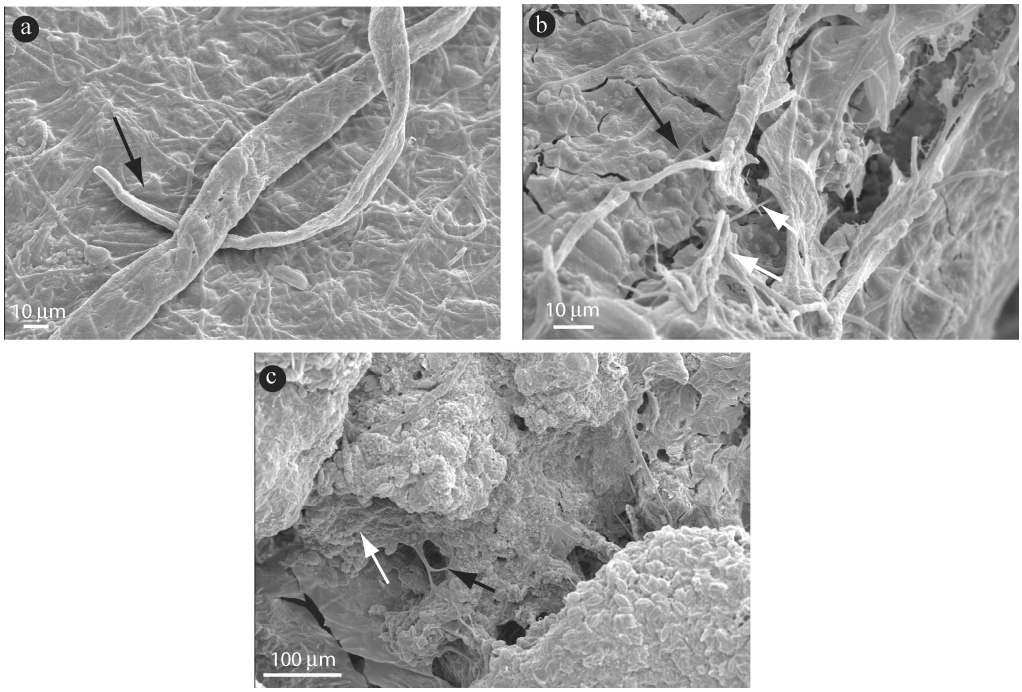

FIG. 7. Mechanical behaviour of recent EPS in a cyanobacterial biofilm *without acid etching* (SEM pictures): (a) constraint-resistant, dense mass of EPS and bacteria (black arrow); (b) dendroid network of the last generation of EPS (white arrow) overlying the dense EPS and bacterial mass (black arrow); (c) EPS alveolar network (white arrow) resulting from high constraints associated with dense EPS and bacterial mass (black arrow).

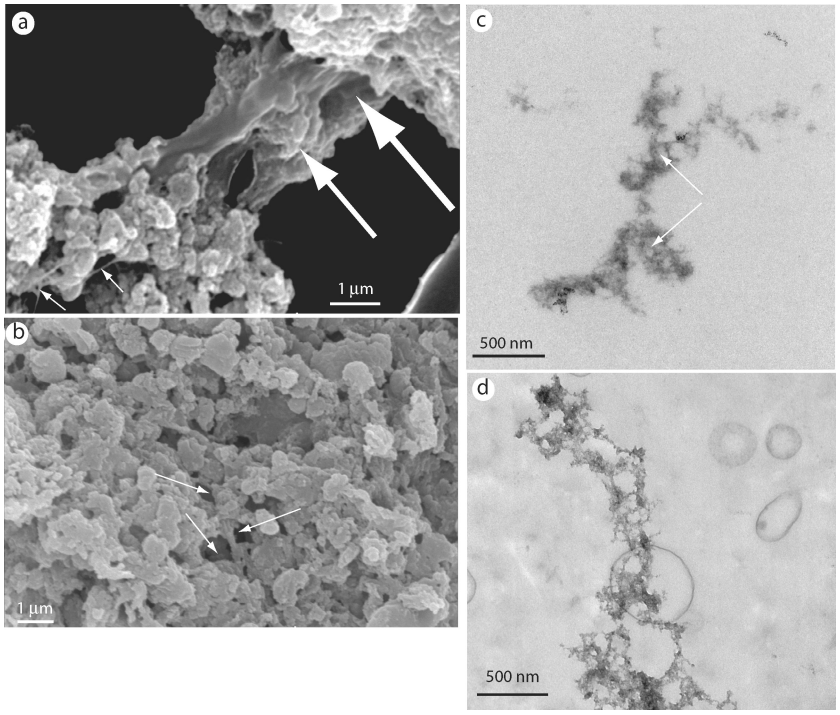

FIG. 8. Fossil amorphous organic matter (AOM) from the Miocene Monterey Formation: (a) agglomerated filaments (big arrow) and dendroid single filaments (small arrow, SEM picture); (b) alveoli (SEM picture); (c) fluffy alveolar network in TEM, ultrathin section; (d) EPS in recent sulphur-oxidizing bacteria in TEM, ultrathin section.

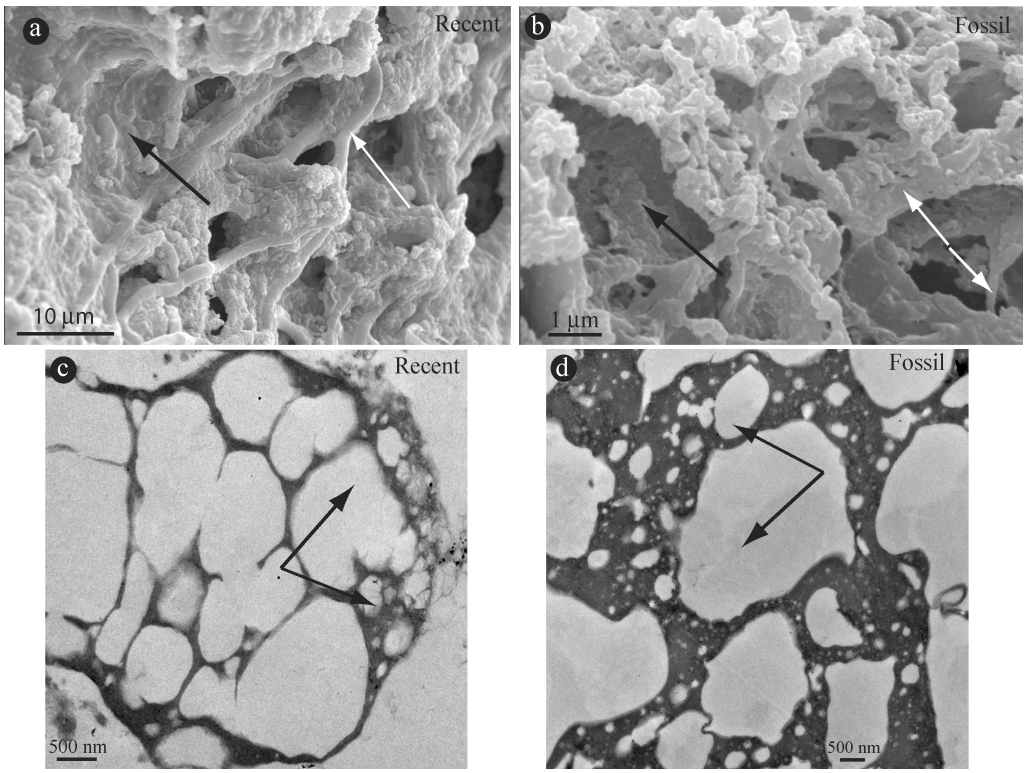

FIG. 9. Comparison of recent cyanobacterial biofilm (a and c) with fossil AOM from Kimmeridgian bituminous shales (b and d) with: (a) repartition of EPS (black arrows) and bacteria (white arrows) in recent biofilm (SEM picture); (b) architecture of fossil OM (SEM picture); (c) recent biofilm with alveolar network (black arrows) in TEM, ultrathin section; (d) alveolar network (black arrows) in fossil OM with different orders of magnitude in fossil OM in TEM, ultrathin section.

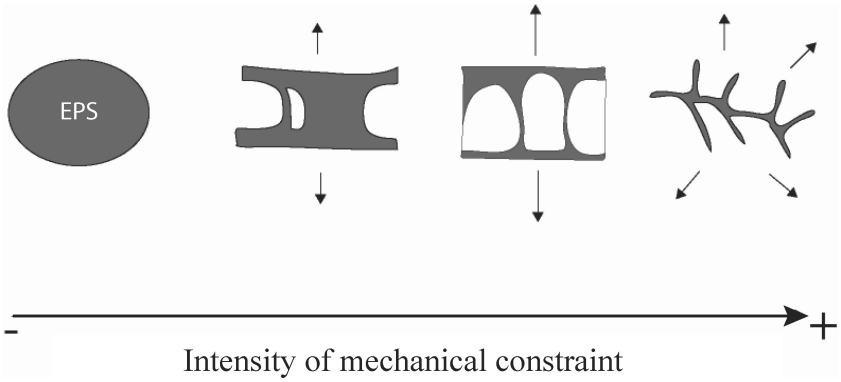

FIG. 10. Model of EPS evolution through time in sulphur-oxidizing bacteria, as a function of mechanical constraints (agglomeration of bacteria and detrital particles, or compaction through diagenesis).

DISCUSSION

In both recent cases studied, EPS seem to condition the biofilm evolution. Indeed, the EPS chemical nature has an impact on the entire biofilm structure and provides it with resistive properties. Based on observations made on sulphuroxidizing bacteria, a model of EPS evolution through time is proposed (Figure 10). It relies on mechanical constraints such as the agglomeration of material (bacteria and detrital particles) and possibly compaction associated with diagenesis.

This behavior is less expressed in the case of a cyanobacterial biofilm. Two hypotheses could be proposed for this. First, the biofilm is constituted of different bacterial populations inducing variations in polysaccharide composition (Jorand et al. 1998; Sutherland 2001), which may create a competition between each EPS type. Each specific secretion reinforces the biofilm structure. Second, the intensity of mechanical constraints may be an important factor in the biofilm cohesion. This intensity may depend on bacterial density and/or the EPS resistance to internal mechanical and chemical constraints related to their chemical nature. It is known that the EPS influence the mechanical stability of recent biofilms (Applegate et al. 1991; Mayer et al. 1999).

In fossil OM, different types of EPS are recognized with respect to the source bacteria. In the Miocene Monterey Formation, EPS in TEM have a typical alveolar network, seem to be constituents of their own and are not a major constituent of AOM. In the Kimmeridgian bituminous shales, the whole OM is characterized by alveoli of different sizes. A good morphological correlation can be established between this fossil OM and a recent cyanobacterial biofilm. Pacton et al. (2006) have already demonstrated that AOM in these bituminous shales is not amorphous but nanostructured. The observations presented here show that EPS probably play a major role in this OM preservation. EPS cannot be detected by organic geochemical methods because of the highly hydrated structure of EPS which become more refractory during degradation (Decho et al. 2005).

In AFM, the nanostructure of Kimmeridgian OM shows smooth areas and alveoli resembling those observed in the recent biofilm. These characteristics illustrate the active role of EPS in the structuration of organic particles through diagenesis. In the Miocene of the Monterey Formation the contribution of EPS in OM preservation seems to be minor: OM is locally characterized by filaments and an altered alveolar network related to stretching.

CONCLUSIONS

In summary, EPS enhance preservation properties in some types of fossil AOM, acting as a protective agent in the sorptive preservation pathway. Their contribution depends on the stability and the chemical composition of the biofilm. This is particularly visible in cyanobacterial biofilms (e.g., in the Kimmeridgian bituminous shales at Orbagnoux). The alveolar network observed in fossil OM is due to the EPS contraction resulting from mechanical constraints, as supported by studies of recent cyanobacterial biofilms. The amount and chemical nature of EPS condition the structuration of OM. Therefore, in the preservation processes, EPS must be considered as a pathway of their own, because they protect OM from degradation and remineralization. This study illustrates the importance of combining optical and chemical analyses when investigating OM preservation pathways. Organic geochemistry cannot detect their presence because they are highly hydrated structures. So far, bacteria have always been considered as agents of OM degradation. The results presented here point to the contrary and highlight that bacterial activity could be more of a preservation than a degradation agent in OM fossilization.

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