Exquisite preservation of a widespread filamentous microorganism in French Cretaceous ambers: Crucial for revising a controversial fossil

Préservation exceptionnelle d’un microorganisme filamentaire dans les ambrés crétacés de France : une clé pour la compréhension d’un fossile controversé

Jean-Paul Saint Martin, Simona Saint Martin

UMR 7207 CR2P MNHN, CNRS, UPMC Sorbonne Université, Centre de recherches sur la paléobiodiversité et les paléoenvironnements, Muséum national d’histoire naturelle, département “Origines et Évolution”, 8, rue Buffon, 75231 Paris cedex 05, France

ARTICLE INFO

Article history:
Received 7 September 2016
Accepted after revision 30 November 2016
Available online 6 July 2017

Keywords:
Amber
Sheathed bacteria
Leptotrichites
Leptothrix
Ultrastructure
Taphonomy
Cretaceous

ABSTRACT

Cretaceous ambers from different localities often contain abundant filamentous microorganisms that extend from the surface of the lumps of amber towards their center. These microfossils have been interpreted in the past as sheathed bacteria, cyanobacteria, and fungal hyphae, respectively. Here, we applied various techniques such as optical microscopy, confocal microscopy, and SEM to constrain the actual nature of these microorganisms. We evaluate published views and new evidence and conclude that the observed morphological and ultrastructural features correspond to sheathed bacteria. We propose a scenario explaining the observed differential preservations as various stages of the sheath construction around the bacterial filaments growing in the resin and the consequences of the transformation of the resin to amber. We suggest an abundant occurrence of at least one extinct resinicolous Leptothrix-like taxon in the Cretaceous Period.

© 2017 Académie des sciences. Published by Elsevier Masson SAS. All rights reserved.

RÉSUMÉ

L’ambre du Crétacé de différentes provenances contient souvent d’abondants microorganismes filamentaires, qui se développent depuis la surface vers le centre des pièces d’ambre. Ces microfossiles ont été interprétés comme des champignons, des cyanobactéries ou des bactéries. Grâce à l’utilisation de diverses techniques d’observation (microscopie optique, microscopie confocale, MEB), il est possible de mieux comprendre la nature de ces microorganismes. L’évaluation des travaux publiés et les nouvelles données obtenues permettent de conclure que les caractéristiques morphologiques et ultrastructurales correspondent à une bactérie à gaine de type Leptothrix. Les préservations différentielles observées sont rapportées aux différentes étapes de la construction de la gaine de filaments bactériens se développant dans la résine et les conséquences de la transformation de la résine en ambre. Ce type de microorganisme semble avoir disparu de la niche résinicole après le Crétacé.


* Corresponding author.
E-mail address: jean-paul.saint-martin@mnhn.fr (J.-P. Saint Martin).

https://doi.org/10.1016/j.crpv.2017.05.003
1631-0683/© 2017 Académie des sciences. Published by Elsevier Masson SAS. All rights reserved.
1. Introduction

Amber acts as a particularly favourable medium for the exceptional preservation of organic inclusions. Arthropods trapped in amber have been the subject of countless works. In contrast, the world of microorganisms was only discussed in detail later. Preservation of microorganism communities in amber is known from the Carboniferous onwards in many deposits the world over (Girard, 2010). Nevertheless, difficulties of approach and inaccuracies result from observational limits imposed by the size of the objects and the identifications based on morphological characters, which are often unclear when only classical optical microscopy is used.

This micro-world entrapped in amber comprises many filamentous microorganisms such as bacteria (including actinomycetes), cyanobacteria and fungi (see review in Saint Martin et al., 2012). Some microorganisms can be considered as resincolonizing colonizers (Reimforde and Schmidt, 2011; Breton, 2012; Breton et al., 2013; Girard, 2010; Saint Martin et al., 2012, 2013; Speranza et al., 2015), presumably using the resin as a nutrient substrate functioning like a culture medium (Breton, 2011). Among these, a particular type of inclusion consists of networks of regular tubular filaments, slightly sinuous, exhibiting a distinct central lumen under the optical microscope. The diameter of filaments ranges between 6 to 10 μm while the lumen diameter is approximately 1 μm. The network develops mostly at the periphery of amber pebbles and more rarely invades the nodule from the outer to the inner part of the fossil resin. This filamentous network was first observed in Albian–Cenomanian amber from Kansas and assigned to the extant sheathed bacterium Leptothrix (Waggoner, 1996). Later, the fossil species Leptothrixites resinatus Schmidt 2005 was defined by Schmidt and Schäfer (2005) as a sheathed filamentous prokaryotic inclusion abundant in the Cretaceous amber of Schliersee (Germany) and considered to be close to the extant genus Leptothrix. Micro-inclusions resembling and/or attributed to L. resinatus were also identified in some Cretaceous ambers from France (Breton, 2012; Girard et al., 2009a; Girard, 2010; Girard et al., 2013a; Néraudeau et al., 2016; Saint Martin et al., 2012, 2013), England (Brasier et al., 2009), Spain and Japan (Beimforde and Schmidt, 2011) and were considered to be prokaryotic remains. In other cases, similar sheathed filamentous inclusions were tentatively assigned to a cyanobacterium and designated as the new fossil species Paleocolteronema cenomanensis Breton and Tostain, 2005 (Breton, 2007; Breton and Tostain, 2005; Breton et al., 2013; Girard, 2010; Girard et al., 2009a, 2013b). However, the same kind of micro-inclusions described in Cretaceous amber from Spain were instead considered to represent fungal hyphae (Ascaso et al., 2003, 2005; Martín-González et al., 2009; Speranza et al., 2010, 2015). The cylindrical structures around the lumen are therefore differently interpreted: as the sheaths of two types of filamentous prokaryotes (bacteria and/or cyanobacteria) or as the cell wall of eukaryotic mycelia (fungi). For reliable identification physical and chemical approaches were performed based on the putative presence of remains of chitin and EPS (Speranza et al., 2015) or phycocyanin pigment (Girard et al., 2009a), the authors recommending the systematic use of their own methods as discriminating. However, such methods are difficult to implement for each amber sample and assume the same degree of preservation of the organic substances despite quite variable taphonomic and diagenetic histories of the original resin.

To overcome the uncertainties about this controversial filamentous organism and to allow a better understanding of the morphology of microorganisms and their mode of preservation, we examined numerous French Cretaceous ambers from various sites and of diverse ages. We paid particularly attention to the finest structures and to the original organic compounds more or less preserved. Following the methodology of Ascaso et al. (2003), we applied various methods of investigations such as classical optical microscopy, Scanning Electron Microscope (SEM) and Confocal Laser Scanning Microscope (CLSM). Thus, the objective of this study is:

• to update the knowledge with complementary observations and data, providing extensive illustrations;
• to account for the mode and the quality of preservation;
• to underline the need to implement various investigative techniques on the same amber samples for better observation and identification of microorganisms, in order to avoid the problem of preservation bias.

2. Material and methods

2.1. Provenance of studied samples

Cretaceous ambers are widely distributed in France. Lacroix (1910) carried out a broad survey of the amber-bearing sites, which was recently updated (Nel et al., 2004; Perrichot and Néraudeau, 2014; Perrichot et al., 2007; Ragazzi et al., 2009), many deposits not being currently accessible. Our observations are based on samples collected from various amber-bearing deposits all over France, ranging in age from Albian to Campanian (Fig. 1, Table 1). According to Nohra et al. (2015), chemical characterization indicates that French Cretaceous ambers are produced by conifers belonging to diverse families.

In most cases, we collected in situ amber samples. We paid particular attention to samples from sites in Charente-Maritime (Archingeay, Cadeuil, Puy-Puy) (Girard, 2010) remarkable for the exceptional preservation of filamentous microstructures.

For comparison, extant samples of Leptothrix were collected from a natural water trickle.

2.2. Microscopic analyses

For optical and confocal microscopy, petrographic thin sections of approximately 30 microns standard thickness, covered with a cover-slip, were made.

About 60 thin sections were examined with an optical microscope (Zeiss Axioscope 40 equipped with a photographic device) provided with ×40, ×63, and ×100 immersion objectives. For the images taken at several focus levels, a specific image processing software (Helicon Focus®) was used.
be obtained by acquisition of stacks of closely spaced sections. CLSM techniques were successfully used in the study of microorganisms in amber (Ascaso et al., 2003, 2005; Martin-González et al., 2009; Saint Martin et al., 2013; Speranza et al., 2010, 2015) or other organic remains such as spider webs (Saint Martin et al., 2014). The CLSM observations were essentially performed on a Leica TCS SP5 microscope (upright and inverted) at the “Université Pierre-et-Marie-Curie”. Some observations were also conducted with the Zeiss LSM700 microscope at the University Aix-Marseille. Samples were observed through $20 \times 0.7$ NA, $40 \times 1.25–0.7$ NA and $63 \times 1.40$ NA oil-immersion objectives. For spectral analysis, the excitation wavelength was 405 nm and the emission was monitored in the 415 to 765 nm range with a band width of 10 nm. The images obtained from confocal microscopy allow viewing of the autofluorescent objects in false colours. 3D reconstruction was obtained by acquisition of stacks of closely spaced confocal optical sections at 0.10 µm to 0.20 µm intervals that guarantee the finest resolution. These images are treated with the open source software ImageJ (Rasband, 1997–2013) for the optimization of the 3D data.

Examinations with Scanning Electron Microscope (SEM) were performed using three SEM equipments: JEOL JCM-6000 NeoScope mainly for exploration, XL 30 ESEM Philips and Hitachi SU3500 for better magnifications, both of which provide Energy Dispersive Spectrum XRay (EDS) micro-analysis. For this purpose, pieces of amber were broken and the fracture surfaces immediately coated with gold-palladium. This method allows the examination of fresh surfaces with minimal risk of contamination (Girard et al., 2009b). The collected samples of Leptothrix were washed with sterilized water and then vacuum-dried. The dried samples were mounted onto a stub and coated with gold-palladium.

### 3. Results

#### 3.1. General description of filaments in amber

The filamentous networks studied herein occur in different types of amber grains (Fig. 2) as described from other Cretaceous ambers (Girard, 2010; Speranza et al., 2015). In all cases, they impart an opaque appearance to the invaded portion of the piece of amber. They often constitute thick opaque cortices (up to 1 cm thick) ranging in color from milky white to brown around drop-shaped or nodular grains (Fig. 3A–G). They can also entirely occupy drop-shaped grains or more complex and larger nodules (Fig. 3H–K).

In the case of the presence of cortex at the periphery of the amber grains, the filament network is centripetally oriented. The growth of filaments is therefore oriented from the surface into the core, the density of filaments being higher at the periphery (Fig. 3C, E, G).

In composite nodules, often consisting of several resin flows, the filament network may be partially distorted by the flow. However, it is frequently observed that the filaments cross the resin flow, which demonstrates colonization of a still liquid resin (Fig. 3J–K).

---

![Figure 1](image.png)

**Fig. 1.** Geographic location of studied amber-bearing deposits in France. **Fig. 1.** Situation géographique des dépôts à ambre de France étudiés.

<table>
<thead>
<tr>
<th>Provenance</th>
<th>Age</th>
<th>Main references about amber sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vendée: La Garnache</td>
<td>Middle</td>
<td>Saint Martin et al., 2014; Néraudeau and Perrichot, 2014</td>
</tr>
<tr>
<td></td>
<td>Cenomanian-Early</td>
<td>Néraudeau et al., 2017</td>
</tr>
<tr>
<td></td>
<td>Santonian</td>
<td>Néraudeau et al., 2002, 2008; Perrichot, 2004, 2005; Girard et al., 2009a, 2009b, 2009c; Girard, 2010; Adi et al., 2011</td>
</tr>
<tr>
<td>Charente-Maritime: Cadeuil, Archingeay, Puy-Puy, Oléron</td>
<td>Upper</td>
<td>Néraudeau et al., 2002; Girard et al., 2009a, 2009b, 2009c; Girard, 2010; Adi et al., 2011</td>
</tr>
<tr>
<td></td>
<td>Albian-Lower</td>
<td>Néraudeau et al., 2002, 2008; Perrichot, 2004, 2005; Girard et al., 2009a, 2009b, 2009c; Girard, 2010; Adi et al., 2011</td>
</tr>
<tr>
<td></td>
<td>Cenomanian</td>
<td>Néraudeau et al., 2016</td>
</tr>
<tr>
<td>Dordogne: Sainte-Mondane</td>
<td>Turonian</td>
<td>Néraudeau et al., 2016</td>
</tr>
<tr>
<td>Aude: Fourtou</td>
<td>Cenomanian</td>
<td>Breton, 2012; Girard et al., 2013a, 2013b</td>
</tr>
<tr>
<td>Ariège: Mas d'Azil</td>
<td>Campanian</td>
<td>Breton et al., 2013</td>
</tr>
<tr>
<td>Bouches-du-Rhône: Martigues, Ensues, Belcodène, Sainte-Baume</td>
<td>Santonian</td>
<td>Guillaume et al., 2006; Onoratini et al., 2009a; Saint Martin et al., 2012; Saint Martin et al., 2013</td>
</tr>
<tr>
<td>Alpes-de-Haute-Provence: Aubignosc, Salignac</td>
<td>Albian</td>
<td>Onoratini et al., 2009a, 2009b</td>
</tr>
<tr>
<td>Vaucluse: Piolenc</td>
<td>Santonian</td>
<td>Gomez et al., 2003</td>
</tr>
</tbody>
</table>
3.2. Main morphological features

The studied filamentous microorganisms in French Cretaceous amber generally exhibit the consortium of characters previously indicated by the different authors mentioned above, especially regarding the dimensions and shapes of the filaments. Entire filaments can reach up to a few millimeters in length. Light microscope examination revealed that a filament comprises an empty core (lumen) and a cylindrical envelope (coat or “sheath”) that appears like a halo surrounding the lumen. The total diameter varies between 4 and 10 μm, depending on the layers that form the coat/sheath. In contrast, the lumen diameter is fairly constant between 1 and 1.5 μm. In detail, the filament can show thickening and constrictions sometimes giving it a puffy appearance. The filaments are characterized by a true dichotomous branching, usually marked by a slight swelling. The branching angles usually range between 50° and 90°. The distance between the branches is quite variable or irregular, depending on the samples and the location in the network.

Despite generally common features, the appearance of the filament network varies optically according to the samples or even within a single amber grain as observed by Breton (2007). The confocal microscopy supports the morphological observations due to the natural fluorescence of some constituents of the preserved filaments and the visualization in space (3D restitution) of filament structures.
Fig. 3. General features of the studied filamentous microorganism. A–G. Development of filamentous network in the cortex (arrow) of drop-shaped grains or nodules observed in optical microscopy (A, C, E, G) and SEM (B, D, F); note the centripetal growth from the periphery towards the interior. H–K. Entire colonization of amber nodules by filamentous networks observed in SEM microscopy (H, I), CLSM technique (J) and optical microscopy (K). Note the development of filamentous network intersecting the resin flow.

Fig. 3. Caractéristiques générales des microorganismes filamentieux étudiés.

(Figs. 4 and 5). However, the fluorescence signal can also vary greatly, or even be absent, depending upon the samples. In order to better understand these different cases, we examined numerous samples under SEM. Such examination is an interesting spatial exercise on the theme “to be or not to be” applied to preservation of structures as voids, internal molds, external molds (or imprints) and casts (Fig. 6).

The different methods of observation highlighted the structural elements of the filamentous network, at various scales (Fig. 7). The lumen (lu) clearly and constantly appears as a void (Fig. 7A–K). Around the lumen a sheath-like envelope is constituted (when present) of successive layers (Fig. 7A–E): the first layer (L1) about 5 to 10 μm in diameter, can sometimes be subdivided into several parts only distinguishable in optical and confocal microscopy.
Filamentous network views with CLSM technique. A–G. Archingeay. H–I. Puy-Puy. Note the intertwined mass of filaments exhibiting true branching (A–C, E–I). Transversal sections (E–D) show the central lumen (arrow) without fluorescence. The external aspects of the sheath are variable: smooth (E, I), dusty to granulose (A–D, F–H). Sparse to dense hair-like projections are clearly distinguishable (D, F, G), sometimes globular (D). The sheath may be constituted by several layers (E) here revealed in red and green false colors.

(Fig. 4) Réseaux filamentieux vus en microscopie confocale (CLSM).
Careful examination of several thin sections and amber pieces allows us to distinguish, often in the same network, many cases of preservation and filament development that exhibit either only some of the layers, or the totality of layers described above:

- lumen apparently without envelope or with poorly defined shadow sheath–like envelope (Figs. 3E, G and 9A);
- lumen surrounded by well-defined smooth “sheath” or dusty to granulose “sheath” (Figs. 3K, 4I, 5H, I, K, M); the “sheath” may be constituted of one or more of the
successive layers (L1 to L4) and can exhibit fragmentations or interruptions (Fig. 4A–D) or variable coarse texture (Figs. 3I–K, 4G);

- lumen surrounded by sparse to dense hair-like projections without clear external delimitation (Figs. 4F–G, 5G, 7I–K, 8D–P);
- lumen surrounded by hair-like projections that are partially coated by a thin layer (Figs. 3D, I and 9F);
- lumen surrounded by radial hair-like projections continuously coated by a more or less thick outer layer (L2), these structures being clearly separated from the amber matrix (Figs. 3F, 6G–J, 7A–C); the radial projections may become irregular, sometimes globular (Fig. 4D);
- tubular structure (lumen + layer L1) released from the amber matrix, observed under SEM (Figs. 6A–J, 7F–H, 8Q–R).
Optical and SEM investigations revealed the presence of minute bubbles varying in size and shape in relation to the filaments (Fig. 9). The bubbles are distinctly defined by a very thin outline (Fig. 9M, O–S), intersecting all visible structures. They can thus occupy a portion of the lumen and the envelope of hair-like expansions or only the external part of the filament. Although generally globular (subspherical) they can take on an oval shape. In some
cases, they follow each other along the lumen and form a funnel alignment (Fig. 9K, M, P, R). Their size ranges from a few microns to tens of microns. Similar bubbles were already described from different ambers and interpreted in different ways: hyphal buds (Waggoner, 1994; Speranza et al., 2015), liquid bubbles resulting from filaments growing in freshwater and trapped by resin flow (Breton and Tostain, 2005; Girard et al., 2009a; Girard, 2010; Girard et al., 2013b) or emanations from metabolic activity (Beimforde and Schmidt, 2011).
4. Discussion

4.1. Identification and comparisons

4.1.1. Three hypotheses for a unique resinicolous inclusion?

Paradoxically, organisms exquisitely preserved in amber with their micro- and nanostructures can cause a real problem of identification. The same type of filamentous networks mentioned here were formally described as different entities, divided among prokaryotes (Breton and Tostain, 2005; Schmidt and Schäfer, 2005) and eukaryotes (Speranza et al., 2015). Despite the divergence of opinions on the nature of these micro-inclusions, considering the similarity between all the records, we strongly suspect that these filamentous inclusions likely correspond to a unique
type of resinicolous microorganism, as previously emphasized (Breton, 2007; Speranza et al., 2015). In light of our findings, we are able to bring evidence for discussing the nature of the filaments involved.

4.1.2. The fungal hypothesis

Speranza et al. (2015) described filaments from Cretaceous Spanish amber, with a diameter of about 5–8 μm that consist of an empty core (diameter 1–2 μm) and a halo (thickness 2–3 μm). They attributed them to filamentous fungi although specific morphological features discriminating fungi (hyphal anastomosis, septa structures, spores and fruiting bodies) are often absent, making the distinction from prokaryotes difficult.

Using the staining technique Calco–Fluor–White (CFW) and Wheat–Germ–Agglutinin conjugated with Fluorescein–Isothiocyanate (WGA-FITC) Speranza et al. (2015) argued that the detection of both beta (1,3)-β-glucans and beta 1,4 linked homopolymer of N-acetylglucosamine should be specific to fungal cell walls. Indeed this technique was used for selective staining of fungal hyphae in plant-fungus association (Meyberg, 1988) for the study of fungal-actinomycete interactions (Wohl and McArther, 2001). But the amino sugar N-acetyl glucosamine (GlcNac) is a well-known component of the cell wall of both Gram-positive and Gram-negative bacteria, algae (especially some diatoms), fungal cell wall chitin, and the extracellular matrix of animal cells (Konopka, 2012; Riemann and Azam, 2002). The bacterium Leptothrix studied by Furutani et al. (2011) in cultures contains saccharic carbohydrates such as glucose, mannose, N-acetyl-galactosamine and N-acetyl-glucosamine in the immature sheath surrounding chained cells that were evidenced by different conjugated lectins (FITC); the N-acetyl-glucosamine was evidenced by wheat germ agglutinin (WGA) lectin. Beta (1,3)-β-glucans are naturally occurring polysaccharides found in both eukaryotes and prokaryotes; they are integral cell wall constituents in fungi, yeasts, some grains and component of extracellular polysaccharides (EPS) produced by several bacteria, (Laroche and Michaud, 2007) and also a major storage polysaccharide in the euglenoids, brown algae, diatoms and chrysophytes (Zekovic et al., 2005). For instance diatoms contains both N-acetyl-glucosamine and beta 1,3 glucans; some bacteria containing N-acetyl-glucosamine may produce EPS containing beta 1,3 glucans. So, apparently, the presence of 1,3 glucan and beta 1,4 linked homopolymer of N-acetylglucosamine seems not to be unequivocally reserved to fungi. In almost all our studied samples, we observed a natural autofluorescence preserved in the filamentous network. We have obtained exactly the same CLSM image showed by Speranza et al. (2015), but without any coloration or treatment (Figs. 4A and 7E). Our results introduce questions about the usefulness of treatment provided by Speranza et al. (2015). Furthermore, the routine use of such an approach ignores the complex reactions that occur during the maturation of the resin and its transformation into amber.

Another unfavorable point for the identification of the structures as fungi is the presence of bubbles around the lumen reported by several authors (see above). The bubbles spread clearly throughout and beyond the lumen and the cylindrical envelope. In the case of the fungal hypothesis, it is very difficult to explain such emanation inside a fungal cell wall.

It also worth noting that the large ratio between the size of the cellular structures (lumen diameter) and the thickness of the supposed cell wall does not match the common characteristics of a fungal filament. Generally, fungi do not provide such thick cell walls being up to 5 μm thick. In order to explain this abnormality, Speranza et al. (2015) only refer to data concerning yeasts, the nature of which seems to be unrelated to the filamentous fungi expected to develop in the resin.

Generally, fungal mycelia identified in amber show characteristics under the optical microscope such as septa, yellow-orange in color, clearly defined cell wall and presence of conidia or spores (Beimforde et al., 2011; Girard, 2010; Néraudeau et al., 2016; Saint Martin et al., 2012, 2013; Schmidt et al., 2014). Moreover, fungal hyphae appear dark outside (due to the cell wall) and bright inside, which contrasts with the bacterial filaments observed here due to the absence of a cell wall.

Most frequently, the fungal mycelia preserved in French amber show no fluorescence, which contrasts sharply with the autofluorescent filaments described in this work, especially when occurring in the same area (Fig. 10A). In very rare cases, the fungal mycelia, however, highlight an auto-fluorescence that evidence the usual proportions between the cell wall thickness and the diameter inside of the filament (Fig. 10B). In this latter case, we can also remark the discrepancy between the appearance of the fungal hyphae and morphology and features of our “sheathed” filaments present in the same sample (Fig. 4B).

Another problem is the nature of the matter, which should, according Speranza et al. (2015), differentiate the suspected fungal cell wall from the surrounding amber. In fact, all our SEM observations (Fig. 6) demonstrate the same identity and continuity of the matter constituting the filamentous morphology and the amber; moreover, this continuity is clearly expressed by the same fine texture visible at high magnification. The continuous nature of matter evidenced in our material is consistent with the images provided by Speranza et al. (2015) themselves in their Fig. 6A, B. To assess the microscopic observations, we performed EDS SEM analyzes that show unambiguously the perfect identity between the matter of the different parts (Fig. 11). Thus, in our opinion there is no real distinction between amber and the supposed fungal cell wall of Speranza et al. (2015).

Considering the geological time scale, numerous studies on worldwide ambers evidence the presence of resinicolous fungal hyphae (growing especially in resin) after the Cretaceous–Paleocene boundary (Rikkinen, 1999, 2003; Rikkinen et al., 2014, 2016; Tuovila et al., 2013). Tuovila et al. (2013) suggested that the resin acting as a special ecological niche was only occupied by prokaryotes in the Mesozoic.

4.1.3. The cyanobacterial hypothesis

The cyanobacterial hypothesis was first proposed by Breton and Tostain (2005), who described a new fossil
cyanobacterium *P. cenomanensis* preserved in Cenomanian amber from Sarthe (France). According to these authors, the filamentous microorganism consists of uniseriate trichomes with cells of 1.5–2.5 μm diameter surrounded by a transparent cylindrical sheath about 8–11 μm in diameter, often with concentric layers, the outer one being thinnest. True dichotomous ramifications occur. Later, several workers mentioned the occurrence of *P. cenomanensis* in different Cretaceous ambers from France (Breton, 2007; Girard et al., 2009a; Girard, 2010; Girard et al., 2013b).

The identification as *Palaeocolteronema* refers to the poorly known extant genus *Colteronema*. The single species belonging to this genus, *Colteronema funebre*, was first described by Copeland (1936) and is known only from thermal assemblages in Yellowstone National Park (USA). A more recent taxonomic revision is available from Komárek et al. (2014). The diameter of the putative trichome of the filaments attributed to *P. cenomanensis* seem very small compared with the diameter (3–5 μm) of extant *Colteronema*. Likewise, *Colteronema* is characterized by a sheath constituted of divergent lamellae. The different photographic illustrations of *P. cenomanensis* do not clearly exhibit this same feature (e.g., Breton and Tostain, 2005; Girard et al., 2009a, 2013b).

Girard et al. (2009a) proposed a new method based on the detection of phycocyanin by fluorescence in order to discriminate the cyanobacterium from sheathed bacterium in Cretaceous ambers. They applied this method to apparently similar filamentous networks developed in Archingeay and Fourtou ambers. Finally, these authors concluded that the filamentous networks were cyanobacteria in Archingeay amber and presumed bacterium *Leptothrix* in Fourtou amber. Nevertheless, Beimforde and Schmidt (2011) expressed doubts about this method since there is no explanation for the exclusion of the measurement of amber fluorescence itself. The same criticism about the measurements of phycocyanin was advanced by Speranza et al. (2015) who underline that there is a serious limitation to the method since the technique applied was destructive implying the mixture of amber and filaments. Our recent SEM observations of amber pieces from Archingeay (Figs. 7B, 8R, 9R) and Fourtou (Figs. 8R and 9N), especially with hair-like expansions and the presence of bubbles, finally demonstrate that the same microorganism occur at both the sites. Moreover, it is stressed that hair-like structures are not known in any cyanobacteria sheath.

Considering several examinations of modern resins, Beimforde and Schmidt (2011) attested to never having observed cyanobacteria growing in the resin. They only rarely observed modern filamentous cyanobacteria enclosed by fresh resin that are able to survive a few hours or days after being embedded.

According to all these observations and remarks, it seems that the cyanobacterial hypothesis is indeed questionable.

### 4.1.4. The bacterial hypothesis

The filaments presented in this study are closely similar to those described as *L. resina* and compared to actual sheathed bacteria *Leptothrix* by Schmidt and Schäfer (2005). The *L. resina* specimens consist of filaments of about 7 to 10 μm in diameter composed generally of a superficially rough sheath and a small central lumen of about 1 μm diameter. The filaments are branched and the branching angles range between 30 and 130 degrees. In some cases, a chain of rod-shaped cells is visible in the center. The SEM images exhibiting external molds of the filaments are very similar to our image data from French amber (Figs. 6 and 7). Later, Beimforde and Schmidt (2011) found filaments comparable to modern *Leptothrix*, in Albian amber pieces from Santander (Spain), exhibiting different stages of preservation: rod-shaped cells, empty sheaths, slightly developed sheath and “fossilized sheaths” illustrated under light (Fig. 3C–F) and SEM microscopy (Fig. 4A–B). Examination of these images reveals that the area corresponding to the “fossilized sheath” exhibits the same morphology with hair-like structures developing from the lumen’s void, as in our material (Figs. 7 and 8). However, in our material we did not really distinguish remains of cells.

The templates of fluorescence obtained from our CLSM observations, fit well with the schemas proposed by...
Fig. 11. EDS SEM analysis of amber samples from Cadeuil and Archingeay showing the same signature for the filamentous structures and the surrounding amber.

Fig. 11. Analyse EDS en microscopie électronique d'échantillons d'ambre de Cadeuil et d'Archingeay montrant la même signature pour les structures filamentueuses et l'ambre encaissant.
Furutani et al. (2011a, 2011b) to describe the different states of the construction of the sheath of the bacteria *Leptothrix* (see below).

The arguments developed by Schmidt and Schäfer (2005) for identification of these fossils as sheathed bacterium comparable to the current *Leptothrix* appear convincing enough that several authors have adopted them (Beimforde and Schmidt, 2011; Breton, 2012; Girard et al., 2009a; Girard, 2010; Girard et al., 2013a; Néraudeau et al., 2016; Saint Martin et al., 2012, 2013). Nevertheless there still remains one morphological difference: the specimens preserved in Cretaceous ambers exhibit real branching while only false branching was observed in cultures for some *Leptothrix* species (Spring, 2006). Another discrepancy concerns the incorporation of Fe/Mn only for extant *Leptothrix* and never for fossil *Leptothrichites*. Our EDX SEM analysis confirms the absence of any iron/Mn encrustations (Fig. 11).

4.1.5. Final regard

Considering all the structural features, it seems more appropriate to conclude that the filamentous microorganism described in this paper is closer to sheathed bacteria such as *Leptothrix*. However, the observed differences still leave room for doubt. It should be noted that this resinicilous microorganism is no longer represented in post-Cretaceous ambers (Tuovila et al., 2013). From another point of view, the presence of radial hair-like structures around the cell string evokes the hair-like ultrastructural features, named pili or fibrilae, of certain infectious non-filamentous bacteria (Proft and Baker, 2009). One can probably appreciate that this is a particular microorganism, which may have affinities with sheathed bacteria but has no extant equivalent. In this sense, we believe that the proposal of Schmidt and Schäfer (2005) to create a new paleontological genus under the name of *Leptothrichites* is a suitable solution. Consequently, we can assign the filamentous microorganism here investigated to *L. resinus*us Schmidt 2005.

4.2. Growing and surviving in resin

Many studies were devoted to modern *Leptothrix*, one of the most widespread sheathed iron bacteria known from freshwater environments with special emphasis on the nature and composition of the extracellular tubular sheath that consists of a network of polysaccharides and protein-rich fibrils (e.g., Emerson and Chiorse, 1993; Chiorse, 1984). More recent publications (e.g., Furutani et al., 2011a, 2011b; Ishihara et al., 2013) explored the formation of the sheath in cultures of strains of *Leptothrix*. These authors wonderfully present schemas of the processes of sheath constructions as following:

- globular secretions from the cell envelope;
- secretion from the cell envelope of thread-type structures that may have swollen tips; these threads occupy the so-called “intervening space” between the cell and the sheath;
- formation of the inner layer of an immature sheath made by an assembly of long fibers;
- thickening of an inner layer constituted of aligned fibrils and heavy deposition of Fe in an outer intermingled fibrous layer.

Interestingly, our observations under SEM and confocal microscopy fit well with this proposed schema for modern *Leptothrix* except for the last step of Fe deposition. But perhaps iron and manganese were absent in the resin. For extant *Leptothrix* these elements derive from the environmental water. So the Cretaceous bacteria inside the resin likely had no or just limited access to Fe/Mn.

We assume that different cases of micro and nanostructures observed in our material may correspond in fact to different states of sheath construction. The sheath of modern sheathed bacteria contains saccharic polymers such as glucose, mannose, galactose, N-acetyl-d-galactosamine and N-acetyl-d-glucosamine detected by fluorescence (Chan et al., 2004; Furutani et al., 2011b). The presence of a natural fluorescence in our material may be explained by the preservation of some of these components. The mapping of fluorescence signal in our material fits well with the morphological features observed in optical microscopy. When present, the first envelope made of successive coats around the core of filament (lumen) may correspond to the intervening space that housed the thread-type structures of *Leptothrix*. This intervening space is probably a liquid phase acting in the continuous transfer of bacterial secretions from the cell to the sheath layers (Furutani et al., 2011a, 2011b). Moreover, according to Ghiorse (1984), these thread-type structures contain acid extracellular polymers. Finally, we can consider that the thread-like structures observed in extant *Leptothrix* correspond to the radial hair-like structures of our fossil material. All these data argue for the initial presence of different substances and secretions whose possible preservation in amber might explain the autofluorescence signal showed in the present study.

Our observations under SEM and confocal microscopy allow us to augment our comprehension of the development of filaments in liquid resin before its solidification and transformation into amber. Modern sheathed bacteria are able to utilize a large variety of organic compounds as sources of carbon: glucose, galactose, fructose, methanol, ethanol, butanol, glycerol, mannitol, acetate, succinate, malate, gluconate, citrate, etc. (Van Veen et al., 1978). Laboratory experiments and field studies carried out by Beimforde and Schmidt (2011) showed that bacteria and fungi actively colonized liquid exudate of *Cycas revoluta* and resin of *Pinus strobus* and sheathed bacteria with sheaths of about 10 microns diameter and a lumen of about 1 micron diameter occur in fresh resin of *Pinus elliotti* in Florida swamp forest. Cretaceous filamentous bacteria could use resin as a favorable environment for growing. We have to keep in mind that amber was initially a resin that experienced successive steps from a liquid phase to a solid one. At the beginning of their development, filaments found resin a natural favorable environment for growing. With ongoing solidification, resin transformed into a hostile environment that challenged the filaments.
to develop survival strategies. Sometimes we observed an autofluorescent supra-layer (L3) recovering the first envelope surrounding the lumen (Fig. 7B and C). We suppose that this supra-layer represents a supplementary extracellular polymeric substance layer secreted by bacterium in response to stress conditions in order to react against desiccation (Roberson and Firestone, 1992). Actualistic studies of EPS from bacterial biofilms evidenced the fluorescence of different compounds of EPS, such as proteins (tyrosine and tryptophan) and humic acids (Bhatia et al., 2013; Chen et al., 2003).

We also observed some variations in the dimensions of sheaths when making comparisons to known extant Leptothrix sheath. This may be explained by the various characteristics liquid resin has. Indeed, microscopic analysis of the formation of sheaths of Leptothrix in culture made by Suzuki et al. (2012) revealed that the thickness of the inner and outer layer of the envelope is influenced by the quality of the growth environment: a nutrient-rich medium activates cell metabolism and favors the formation of a thicker inner sheath wall and thicker outer coat fibrils; the sheath observed in culture medium may be twice as thick as that of the natural environment. Liquid resin may be considered a special culture medium that could induce some modifications in sheath dimensions. Ghiorse (1984) thus noted that the forms of sheaths produced by iron bacteria change under different conditions of culture media.

As already explained above, we did not observe iron or manganese impregnation of the sheaths in any of the studied samples. This might be explained by the state of maturity of sheaths. Rogers and Anderson (1976) showed that the process mediating Fe deposition is independent of the Fe concentration in the media; it appeared to be self-sustaining after certain cellular sheath constituents had been synthesized and deposition was effective at the end of the growth phase of bacterium. We may assume that in the case of filaments occurring in amber, the preserved sheath did not achieve its final state of growth and so was unable to initiate Fe deposition.

4.3. Constructing vs. degrading: two different taphonomic stories

As explained above, the same filamentous structure preserved in diverse amber was interpreted as sheathed bacteria or fungi. These two visions generate two different taphonomic schemas. Speranza et al. (2015) considered the filamentous microorganism preserved in cretaceous amber from Spain to be fungal mycelium and proposed a model of progressive hyphal cell wall degradation. These authors stated that “some previously indicated taxonomic features of this microorganism may actually be fossil diagenetic artefacts”. Their image data (light microscopy and SEM) sustaining the three stages of fungi cell wall degradation are very similar to our own image data of French Cretaceous amber which explains in our opinion different stages of bacterial sheath construction. Of particular interest is the question of “empty radial fibrillar structures” of these authors (Fig. 6A, B in Speranza et al., 2005) quite similar to radial hair-like expansions described in this paper (Figs. 6–8). In both cases the radial structures develop from the void of the lumen; in both cases there can be observed the imprints of the origin of these radial structures appearing as little circular voids on external imprints. Speranza et al. (2005) consider the “empty radial fibrillar structures” to be matrix produced by degradation of structural polysaccharides from the fungal cell wall skeleton appearing after the solidification of resin since they are not infilled with resin. It is generally known that the major components of fungal cell wall are chitin, glucans and glycoproteins that are cross-linked together to constitute a fibrillar network, most of the chitin being located near to the plasma membrane (Bowman and Free, 2006). Because these components are interwoven to form a tangled network, it seems difficult to imagine in our opinion that their degradation could produce radial structures which will cross the whole cell wall as suggested by Speranza et al. (2005). Moreover, the scale of dimensions of these fibrillar structures is a key to the comprehension of what we observe. In our opinion, these empty fibrillar (or more accurately hair-like) structures, represent the external molds of fine morphological features of the filamentous microorganism. As explained above, they may correspond in fact to the thread-like projections produced by a bacterium in order to start the construction of an envelope. The insertion of the hair-like structures is clearly distinguishable in external mold appearing as little round voids of various diameters (300–500 nm) (Fig. 7C and J). Based on their material, Speranza et al. (2005) curiously argue that the peripheral holes in the external surface of the wall correspond to a loss of amber material but they do not really explain this surprising phenomenon. Moreover, Speranza et al. (2015, Fig. 6D–F) present photos of tubular filaments and their external imprints that are identical to our image data (Figs. 6 and 7). Speranza et al. (2015) consider these tubular filaments to be hypha and note the irregular eroded appearance of the wall. From our point of view, this cylinder (tube) represents in fact the geometric volume of the bacterium structure. In fact, we can easily observe the continuity of amber mater from the sheath to the surrounding amber. Besides, EDS SEM analysis does not show significant differences between the sheath (the preserved fungal cell wall for Speranza et al., 2015) and the surrounding amber (Fig. 11). It is a peculiar reasoning to invoke the loss of material (amber): if this really is the case, we must question where the lost material has gone.

The irregular external appearance of filaments suggests a close similarity to the outer coat of modern Leptothrix sheath (Fig. 12) that present tightly agglutinated thin fibrils (Furutani et al., 2011b; Hashimoto et al., 2015; Ishihara et al., 2014). The SEM image data from French amber are also much closer to those presented for the sheathed bacterium L. resinatus by Schmidt and Schäfer (2005) and Beimforde and Schmidt (2011), who interpret the rings of small holes as morphological sheath features.

Generally, the fossil world is revealed by various modes of preservation in rocks: impressions, inner mold, outer mold, casts, all resulting in a set of volume, negatively (holes) or positively (casts, etc.). It is also the case for amber that exhibits the same type of preservation processes. For instance, examination of amber samples under SEM reveals
the molding process (Fig. 13) allowing the preservation of very fine structures of various microorganisms such as dinoflagellates (Masure et al., 2013) or actinomycetes (Néraudeau et al., 2016; Saint Martin et al., 2012). It is this fundamental aspect that strongly separates the two taphonomic stories.

The preservation of the filaments in amber as observed today represents various moments of a complex process involving successive or interactive constraints:

- biological, in relation to the vital functions of living organisms;
- chemical with the original nature of the different compounds, the interactions with the environment (temperature, humidity, seasonal variations…) (Martínez-Delclòs et al., 2004), and the transformations affecting the resin such as polymerization and aromatization (Nohra et al., 2015; Ragazzi et al., 2009);
- physical when hardening affects the resin.

An essential taphonomic aspect which is very difficult to assess concerns the subtle interactions between the resin and the filaments developing inside: how to produce potential osmotic exchanges and what is the result; what is the role of resin impregnation on the structures that the microorganisms implement and, conversely, how extracellular productions such as EPS diffuse from microorganism into the resin itself. Deciphering these intimate phenomena would better contribute to understand the autofluorescence signal. The story continues with burial conditions in the amber-bearing sediments and diagenetic processes that affect them. The complexity of all possible interactions during the whole process partially explains the diversity of preservation estimated from different observation techniques. However the scenario proposed here allows the better integration of all these uncertainties, considering that contrary to assumptions of Speranza et al. (2015) the observed morphological features are not diagenetic artifacts, but correspond to fossilized structures. First, there is the growth of mycelium in a liquid medium in which the flow is variable depending on the type of resin production (location, composition), the timing of the beginning of microbial colonization, the external conditions (temperature, humidity) that induce the gradual hardening of the resin. During this time, living cells will start to produce a sheath according to the stages listed above. Relationship and/or exchanges with ambient resin will likely have an impact on the composition of this sheath, which unfortunately is difficult to decipher in the fossilized material.

During the fossilization process of the sheathed bacterium in amber, the original living matter may disappear, thereby leaving voids. In contrast, it was observed that modern Leptothrix cells can leave the sheath when the environmental conditions become unfavorable. Maybe the cells
just abandoned the resin-impregnated sheath towards the outside of the resin lump when the resin hardened and moved to nearby fresh resin flows, as suggested by Schmidt and Schäfer (2005). Anyway we observe in amber several kinds of voids: the lumen corresponding to the location of cells, the voids of hair-like structures, the void corre-
spending to the outer part of the sheath and, when present, the voids of the fibrous structure of the outer layer. The remaining mass around these different voids consists of the original material of the resin that is, it must be repeated, the growth medium for the microorganism. After hardening
of the resin, these voids express the frame of the initial morphological architecture. In addition, the missing living matter has also left imprints emphasizing ultrastructural features. This helps to explain the observed continuity of amber material between the different parts of the filament structure and the surrounding amber. Indeed, contrary to a degradation scheme (see Fig. 7 in Speranza et al., 2015), this continuity is everywhere visible and even apparent in the SEM representations proposed by these authors.

5. Conclusion

*L. resinatus* appears to be a very common component of the micro-world in French Cretaceous ambers whatever the botanical origin of the resin is, and more generally in worldwide Cretaceous ambers. All previous studies show a remarkable state of preservation that allows detailed inter-
pretation of the structures down to sub-micrometric scale. However, until now this microorganism is still quite enig-
matic since it does not precisely correspond to any known extant microorganism, which led to different identifica-
tions, from different prokaryotes to fungi. One explanation is surely differential preservation depending on different factors in play during the life time of the microorganism and the subsequent taphonomic history of the resin. Our observations do not match the degradation hypothesis of fungal mycelium. As an alternative, we here propose a con-
struction scenario involving the growth of a bacterium and its sheath. Thus, French amber exceptionally registered a suite of life instants in this construction-growth pro-
cess. *L. resinatus* shares morphological and growth features with the extant sheathed bacterium *Leptothrix*. In addition, we noted some differences that make *L. resinatus* a special filamentous resinicollous microorganism, apparently not recorded after the Cretaceous. Provocatively we raise the possibility that the filamentous microorganism named *L. resinatus* preserved only in Cretaceous amber could be one of the numerous victims of the Cretaceous–Cenozoic biological crisis.

Acknowledgments

We address special thanks to Didier Néraudeau (Uni-
versity Rennes 1) for having supported us in our research and for providing amber samples of various origins. We are also indebted to Gérard Breton for kindly providing pieces of Mas d’Azil amber and Luc Ebbo for his invaluable assistance in field. The preparation of thin sections was performed by Lilian Cazes and Séverin Morel (UMR 7207). We thank Roger Notonier and Alain Tonetto (Aix-Marseille
Université) and Catherine Rausch, Cyril Willig, Caroline Dalle and Gérardine Toutoiras (PTME, "Muséum national d’histoire naturelle", Paris) for their assistance with scan-
ing electron microscopy. The main CLSM investigations were performed at the Institute of Biology Paris-Seine Imaging Facility that is strongly supported by the “Con-
seil regional d’Île-de-France”, the French National Research Council (CNRS) and Sorbonne University, UPMC Univ. Paris 06. This paper is a contribution to the specific research programme (ATOM) of the “Muséum national d’histoire naturelle” (Paris) *Les microorganismes, acteurs clés des écosystèmes*. We thank sincerely the two anonym-
ous reviewers for their very constructive remarks that improved the manuscript. We are very grateful to Martin Pickford for improving the English language.

References


Beimforde, C., Schmidt, A.R., 2011. Microbes in resinous habitats: a compila-

Beimforde, C., Schäfer, N., Dörfelt, H., Nascimbeni, P.C., Singh, H., Hein-

Bhatia, D., Bouven, I., Simon, S., Bordas, F., Van Hullebusch, E.D., Rossano, S., Lens, P.N., Guibaud, G., 2013. Fluorescence detection to determine proteins and humic-like substances fingerprints of exopolymeric sub-
stances (EPS) from biological sludges performed by size exclusion chromatography (SEC). Bioresource. Technol. 131, 159–165.


Emerson, D., Ghiorse, W.C., 1993. Ultrastructure and chemical composi-
tion of the sheath of *Leptothrix*. J. Bacteriol. 175 (24), 7808–7818.

Furutani, M., Suzuki, T., Ishihara, H., Hashimoto, H., Kunoh, H., Takada, M., 2011a. Initial assemblage of bacterial saccharic fibrils and ele-
ment deposition to form an immature sheath in cultured *Leptothrix* sp. Strain OUMS1. Minerals 1, 157–166.


