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“Candidatus Thiovulum sp. strain imperiosus”*: The largest free-living epsilon proteobacteriaeota**Thiovulum* strain lives in marine mangrove environment.**

Marie-Noëlle Sylvestre^{a*}, Patrick Jean-Louis^a, Adrien Grimonprez^a, Philippe Bilas^{b, c}, Amandine Collienne^a, Catherine Azède^a, and Olivier Gros^{a, b}

^a Institut de Systématique, Evolution, Biodiversité (ISYEB), Muséum National d'Histoire Naturelle, CNRS, Sorbonne Université, EPHE, Université des Antilles, BP 592 - 97159 Pointe-à-Pitre, Guadeloupe.

^b Centre Commun de Caractérisation des Matériaux des Antilles et de la Guyane (C³MAG), UFR des Sciences Exactes et Naturelles, Université des Antilles, BP 592 - 97159 Pointe-à-Pitre, Guadeloupe.

^c Groupe de Technologie des Surfaces et des Interfaces, Université des Antilles, BP 592 - 97159 Pointe-à-Pitre, Guadeloupe.

* Corresponding author: e-mail address: marie-noelle.sylvestre@univ-antilles.fr

ABSTRACT

A large ($47.75 \pm 3.56 \mu\text{m}$ in diameter) *Thiovulum* bacterial strain forming white veils is described from marine mangrove ecosystem. High sulfide concentrations (up to 8 mM of H_2S) were measured on the sunken organic matters (wood/bone debris) in laboratory conditions. This sulfur-oxidizing bacterium colonized such organic matter forming white veil. According to conventional scanning electron microscope (SEM) observations, bacterial cells are ovoid and slightly motile by numerous small flagella present through the cell surface. Intracytoplasmic large internal sulfur granules were shown suggesting a sulphidic-based metabolism. Observations were confirmed by sulfur elemental sulfur distribution detected by energy-dispersive X-ray spectroscopy (EDXS) analysis using environmental scanning electron microscope (ESEM) on non-dehydrated samples. Phylogenetic analysis of partial sequence of 16S rDNA obtained from purified fractions of this ϵ -proteobacteraeota strain indicates that this bacterium belongs to the *Thiovulaceae* cluster and could be one of the largest *Thiovulum* ever described. We propose to name this species "*Candidatus Thiovulum* sp. strain imperiosus".

Keywords: ultrastructure, EDXS analysis, phylogeny, epsilon proteobacteraeota, Thiovulaceae

RESUME

Nous décrivons ici une nouvelle souche bactérienne du genre *Thiovulum*, de grande taille ($47.75 \pm 3.56 \mu\text{m}$ de diamètre), formant des voiles blancs dans les mangroves de bord de mer. De fortes concentrations de sulfure (jusqu'à 8 mM de H_2S) ont été mesurées sur les matières organiques immergées (débris de bois/d'os) dans des conditions de laboratoire. Cette bactérie qui oxyde le soufre colonise ces matières organiques en formant un voile blanc. D'après les observations effectuées en microscopie électronique à balayage (MEB), ces bactéries sont ovoïdes et mobiles grâce à de nombreux flagelles. Des granules internes de soufre intracytoplasmique ont été mis en évidence, suggérant un métabolisme sulfo-oxydant. Ces observations ont été confirmées par l'analyse de la distribution de soufre élémentaire, détectée par spectroscopie de rayons X à dispersion d'énergie (EDXS) et réalisée à l'aide d'un microscope électronique à balayage environnemental (ESEM) sur des échantillons non déshydratés. L'analyse phylogénétique effectuée à partir de fractions purifiées de cette ϵ -protéobactérie indique que cette nouvelle souche appartiendrait au genre *Thiovulum* et pourrait être le représentant des plus grands *Thiovulum* jamais décrits. Nous proposons de nommer cette espèce "*Candidatus Thiovulum* sp. souche imperiosus".

Mots-clés : ultrastructure, analyse EDXS, phylogénie, epsilon proteobacteraeota, Thiovulaceae

INTRODUCTION

In marine environments, the anaerobic degradation of organic matter by sulfate-reducing bacteria, result in the production of sulfides sustaining the development of various sulfur-oxidizing bacteria. Marine mangroves are conducive to large organic carbon influx from the mangrove forest resulting in almost millimolar sulfide enrichment in the sediment, through anaerobic degradation processes (Laurent et al. 2009). This increase in sulfide supports the development of various bacteria as free-living organisms forming dense bacterial matt of filamentous Beggiatoaceae (Jean et al. 2015) or large veils covering the sediment of small *Thiovulum* strains (Gros 2017). Some of these thioautotrophic bacteria are also involved in symbiotic associations with diverse marine organisms (Frenkiel et al. 1996; Ott et al. 2004; Himmel et al. 2009; Abouna et al. 2015). Epsilon-proteobacterial species represented the fifth validly described class of the phylum Proteobacteria, known to play ecologically important roles across a diverse range of environments. In the latter, they are important chemolithotrophic primary producers either in deep-sea hydrothermal vents (Huber et al. 2010; Flores et al. 2011) or in shallow water marine mangrove (Gros 2017). A recent phylogenomic characterization of several hundreds of available 16S rDNA Epsilon-proteobacteria public sequences has shown that this Epsilon-proteobacteria group should be reassigned to a novel phylum named Epsilonbacteraeota (Waite et al. 2017). This new phylum gathers 12 families including the Thiovulaceae colonizing saline environments (Waite et al. 2017).

In the veils formed by epsilon-proteobacteraeota, many *Thiovulum* communities can coexist, each population exhibit specific levels of organization through nutrient gradients. They could be free-swimming cells or linked-cells attached to each other by a matrix of mucus on top of the sediment. By moving collectively or individually, the rotational movement of cells flagella generate a macroscopic flow across the nutrient gradient (reduced sulfur species, oxygen, CO₂ and potential nitrogen compounds). The stirring of surrounding water enhances oxygen uptake by the bacteria and draws nutrient-rich water to the cells (Wirsen and Jannasch 1978; Petroff and Libchaber 2014, Petroff et al. 2015; Gros 2017; Wait et

al. 2017). The properties of such veils have been summarized recently (Gros 2017). In shallow marine environments, an ability to build conspicuous veils has also been reported for other epsilon-proteobacteria, such as those of the genus *Arcobacter* (Wirsén et al. 2002) and *Candidatus Thioturbo danicus* (Muyzer et al. 2005). Studies concerning epsilon-proteobacteria belonging to the genus *Thiovulum* show that there are very few sequences available (Waite et al. 2017). Recently, the number of sequences available has been improved thanks to a new strain described from marine mangrove in the Caribbean (Gros 2017). However, the knowledge of these poorly studied thioautotrophic bacterial species belonging to the group of epsilon-proteobacteria must be improved to show the contribution of *Thiovulum*-dominated microbial mats to the biochemical cycles and food web of marine mangroves. Moreover, the first and unique single cell genome analysis of *Thiovulum* cells was done ten years ago and revealed common characteristics relative to the genus *Thiovulum* like chemolithoautotrophy metabolism, chemotaxis, and a greater genotypic and phenotypic diversity than expected in the bacterial community according to conventional markers as 16S rDNA sequencing (Marshall et al. 2012).

Colorless sulfur *Beggiatoa* and *Thiovulum* bacteria are often observed either as isolated patches on sediments rich in organic matter located along the protected shores of lakes and the sea or in a transitional niche between O₂ and H₂S in sulfide-rich aquatic environments (Nelson et al. 1986; Thar et al. 2001; Kamp et al. 2006). Two species of benthic Beggiatoaceae (belonging to the genera *Maribeggiatoa* and *Isobeggiatoa*) colonizing marine mangrove sediment adjacent to mangrove roots of *Rhizophora mangle* were described in marine shallow-water environments (Jean 2015). However, no *Thiovulum* veil was observed associated to these mats of Beggiatoaceae. Recently, veils of *Thiovulum* sp. strain karukerense were described as covering marine sediment and/or sunken organic debris in mangrove habitats (Gros 2017). Microbial degradation of organic material in marine environments generates anoxic niches suitable for sulfate-reducing bacteria (SRB) producing large amounts of sulfides diffusing from the sediment to the water column (Leschine 1995; Laurent et al. 2009, 2013). Similar mechanisms occur if the

organic material correspond to bones from dead marine animals known as “whale falls” (Smith and Baco 2003; Treude et al. 2009). As epsilon-proteobacteriaeota metabolism is mainly based on oxidation of sulfides (HS^- , H_2S), high and constant concentration of sulfide was expected in the environment of *Thiovulum* cells. Previous studies have shown that *in situ* measurements of sulfides could be done by using autonomous probes (Laurent et al. 2009, 2013) as well as *ex situ* in the laboratory in mesocosm-like experiments (Jean 2015) in order to detect sulfidic-rich environments.

The presence of various *Thiovulum* species has been described in diverse ecosystems such as: oil well (Gevertz et al. 2000); hydrothermal vents (Moyer et al. 1995; Corre et al. 2001); marine sediments (Madrid et al. 2001); cave springs (Engel et al. 2003); saline environments (Waite et al. 2017) and recently marine mangrove environments (Gros 2017). We provide here the first description of the largest sulfur-oxidizing epsilon-proteobacteriaeota strain (“*Candidatus* *Thiovulum* sp. strain imperiosus”) colonizing organic debris in marine mangrove environments by using a combination of phylogenetic analysis (16S rDNA sequence), ultrastructural investigations (SEM, ESEM, TEM, EDXS) and chemical characterization of the veil environment. This new large marine mangrove strain belonging to the same cluster than *Thiovulum* sp. strain *karukerense*, could represent a new genus of *Thiovulum*. This newly described strain of epsilon-sulfur-oxidizing *Thiovulum* bacterium and its potential interactions in the microbial communities from marine mangrove ecosystem, will be a part of a further metagenomic and single-cell sequencing study.

MATERIALS AND METHODS

Sampling

Cleaned pig bones (heads stripped of their flesh) or freshly cut fractions of branches of the mangrove tree *Rhizophora mangle* were deployed in the marine part of the mangrove of Guadeloupe (N16°/W61.5) at ≈ 1.30 m depth. In order to avoid contact with the organic-rich sulfidic sediment, the

devices were maintained about 20-30 cm above the bottom of sediment using a metallic structure covered by a plastic mesh (1 x 0.5 x 0.5 m).

Biological colonization (presence of a veil covering the sunken organic materials) was monitored regularly by *in situ* and *in vitro* visual inspections. Veils were directly collected from organic materials immersed in water for different time periods with a 60 mL syringe, transported to the laboratory and transferred to a large glass Petri dishes in order to follow and select the colorless bacteria under a binocular microscope. For chemical measurement, some bones were collected after 3 days of deployment, delicately placed with their surrounding water and veil in a container and brought back to the laboratory. Bones, water, and veil were placed in an oxygenated aquarium (Figs. 1A-B). Two days later (i.e. 5 days after deployment), the veil around the bone was reconstituted. Kinetics of production and dissemination of sulfides (H_2S and total $\text{H}_2\text{S} + \text{HS}^-$) and oxygen were monitored inside and outside the veil.

Chemical measurement: Environmental Oxygen and Sulfide measurements

Concentration profiles of oxygen and hydrogen sulfides ($\text{H}_2\text{S} + \text{HS}^-$) were measured continuously during 90 minutes by microsensors probes, positioned respectively outside the veil, i.e. in the 500 to 1100 μm of water surrounding the oxic/anoxic interface and inside the veil. The distances of the microsensors probes from the oxic/anoxic interface were measured with a micromanipulator (MM33-unisens). H_2S present in the medium enters the probe tip, where it is converted into HS^- ions in the alkaline electrolyte. HS^- is then immediately oxidized by the ferricyanide to sulfur and ferrocyanide. The potential difference (mV) generated by the re-oxidation of ferrocyanide, proportional to the partial pressure of H_2S , is recorded by a "Unisense MicroSensor version 2.01 Multimeter," which is connected to the sensors. The fact that microsensors (Unisense Ox-100 and Unisense H_2S -100) measuring oxygen and sulfides, have each been previously calibrated as described by Jean et al. (2015), makes it possible to translate this signal into concentration ($\mu\text{mol.L}^{-1}$) using the "Sensor Trace Basic 3.0" program (Jeroschewski et al. 1996). The

calculation of the total sulfide concentration (H_2S , HS^- , S^{2-}), also considers the pH of the sample (Kühl and Steuckart 2000) and the following balances:

Light Microscopy

Dozens of freshly collected cells were pooled in 500 μl of filtered seawater using a binocular magnifying microscope (Leica MS 5) and killed with one drop of 25% glutaraldehyde solution. *Candidatus* Thiovulum sp. strain imperiosus was then observed and photographed without staining or dehydration processes with a Nomarski Axioskop2 contrast microscope (Figs. 2C-D) and with a Nikon DXM 1200F camera (Fig. 2B). One hundred of cells were measured with the microscope in order to get the average cell size of this new epsilon proteobacteriaeota strain.

Ultrastructural analyses

Scanning Electron Microscope (SEM)

Samples for conventional SEM observations were fixed 2 hours at 4°C in 2.5% glutaraldehyde in 0.2 μm filtered seawater and dropped on silanized (3-aminopropyltriethoxysilane; Sigma-Aldrich, USA) slides. Slides were placed in a humid chamber overnight to allow time for the bacteria to sediment and stick to the surface of the slide under a humid atmosphere. Each slide was rinsed twice in filtered seawater, dehydrated in graded acetone series, critical point dried with CO_2 as transitional fluid (EM CPD300, Leica) and sputter-coated with gold (Sputter coater SC500, Biorad) before observation with FEI Quanta 250 microscope at 20 kV.

Energy-dispersive X-ray spectroscopy (EDXs) analysis

In order to detect elemental compounds from bacterial cells (such as sulfur), freshly fixed bacteria (as described for SEM preparation samples above) were observed using an Environmental Scanning Electron Microscope (FEI Quanta 250) operating at 15 kV under an environmental pressure of 7 Torr at 5°C. Energy-dispersive X-ray (EDX) measurements were conducted with an M-max 50 mm² Oxford detector. Just before observation, samples were rinsed quickly three times with deionized water in order to avoid salts crystallization and placed promptly in the microscope. Elemental mappings were obtained after 180 seconds of acquisition time.

Transmission Electron Microscope (TEM)

For TEM observations, samples were prefixed for one hour at 4°C in 2.5% glutaraldehyde/0.1 M cacodylate buffer (pH 7.2) adjusted to 900 mOsM with NaCl and CaCl₂ in order to improve membrane preservation. Samples were then rinsed twice in 0.1 M sodium cacodylate buffer for 5 minutes in order to remove aldehyde before fixation of 45 minutes at room temperature in 1% osmium tetroxide, in the same buffer, with a final osmolarity adjusted to 1000 mOsM. Then, samples were rinsed three times in distilled water for 5 minutes, and post-fixed with 2% aqueous uranyl acetate for one more hour (Hayat and Giaquinta 1970). After three rinses in distilled water, specimens were dehydrated through a graded acetone series at room temperature before embedding in Epoxy resin (Glauert 1991; Gros et al. 2012). Thin sections (60 nm thick) were observed in a Tecnai G20 TEM at 200 Kv.

Raman spectroscopy analysis

Living individual cells were deposited on a circled section of a slide and were directly analyzed in filtered seawater (0.22m). Seawater constitutes both an isotonic medium for marine micro-organisms and a good cooling medium to avoid irradiation damages of the samples during Raman analyses. Spectroscopy analyses were performed using a HR 800 Horiba multi-channel spectrometer fitted with a Peltier cooled

CCD detector for spectra recording. The exciting monochromatic light used is the 405 nm wavelength line delivered by a solid Nd YAG laser. The objective lens used was X50, the confocal hole was 500 nm. The laser power on the sample was limited to 1 mW to avoid sample damage more over samples were maintained in seawater, which constitutes a good cooling isotonic medium. The acquisition time was 5 seconds, and in order to obtain a good signal-to-noise ratio five spectra were accumulated. The pre-monochromator is a notch filter and the monochromator were a 600 lines/mm grating allowing recording of a one shot spectrum over an energy range of 350 cm^{-1} to 3250 cm^{-1} .

Molecular characterization

DNA extraction: Due to the large size of the cells, 200 bacterial cells were prepared after a meticulous sorting of the bacteria in $0.2\mu\text{m}$ filtered seawater under a light microscope. Then cells were washed three times in sterile sea-water. At each wash, large cells were sorted and they were pipetted individually to avoid contamination with other large bacterial cells like *T. karukerense*. Then, the purified cells were pelleted after a short centrifugation ($4000g$, 2 min) before disruption *via* three repeated cycles of freezing (5 min at -80°C) and thawing (5 min at 90°C) to release DNA which was then extracted using DNeasy Blood & Tissue kit (Qiagen) according to the manufacturer's instructions.

PCR amplification: 16S rDNA was amplified using universal primer sets 8F/907R and 533F/1492R (Lane et al. 1985, 1991; Marchesi et al. 1998). PCR amplifications were performed as previously described (Jean et al. 2015) and PCR products were directly sequenced by Eurofins (Germany) using the Sanger sequencing method.

Phylogenetic analysis: Phylogenetic tree was obtained from 16S rRNA gene sequences of representative species of the *epsilon*-proteobacteriaeota as previously described (Gros 2017). *Agrobacterium tumefaciens* (D14500) was used as outgroup.

RESULTS

Colonization pattern of organic debris deployed by *large colorless* cells

Colonization of organic matter such as plant debris or bones by micro-organisms in mangrove is a multiple-step process involving many bacteria using different metabolisms. The study of the ecological kinetics of free sulfur-oxidizing bacteria and protists populations on *Rhizophora mangle* branches and skulls of pig deployed in mangrove was observed on binocular microscope (magnification Gx40) and was showed on cjm-2021-0101.R1suppla. After 3-4 days, veils collected on the different organic substrates immersed artificially are mainly composed of *Thiovulum* sp. strain karukerense. After 4-5 days, many eukaryotic-microorganisms such as protists, nematodes and crustaceans, which are probably attracted by prey concentration, colonize the veils. Some of them, known to feed on bacteria as ciliates and flagellates ingest *Thiovulum* sp. strain karukerense and other bacteria present in the veil allowing the transfer of microbial biomass to higher trophic level (unpublished data). After 5-7 days, veils observations showed a radical shift on microbe community. Populations of *Thiovulum* sp. strain karukerense, ciliates and flagellates highly decrease and are substituted by the larger colorless bacteria which are described in this study and probably belonging to the *Thiovulum* genus and other protists (cjm-2021-0101.R1suppla). Both *Thiovulum* sp. strains could be present and have been observed in the same veil. However, *Thiovulum* sp. strain karukerense tend to disappear from the veils progressively and is substituted by the larger strain *Candidatus* *Thiovulum* sp. strain imperiosus (Fig.3B). These bacteria are large enough (~50µm) to be harvested under a binocular microscope (Gx40) and thus make it possible to constitute a fraction composed almost exclusively of this characteristic bacterial morphotype.

Sulfide measurements

In order to characterize the veil environment around deployed bones, *ex situ* sulfide measurements were performed in the laboratory the day after collection in the field. A total of 21

independent profiles obtained from 10 different deployed bones (over a period of several months) were obtained. Only the most stable profile (Fig. 1B) obtained from the 21 measured profiles was presented in this study, but the average of the maximum values obtained from these 21 profiles is indicated in brackets. In the 500 to 1100 μm of water surrounding the oxic/anoxic interface of the veil, almost no sulfide was detected, concentration measured ranging from 0 to 18 μM (Fig.1B-red and green lines). The water column was normally oxygenated (Fig.1B-blue line) with a mean concentration of $902.68 \pm 96.73 \mu\text{M}$, ($1600 \pm 168 \mu\text{M}$). Once the sensor penetrates within the veil, sulfide concentrations [H_2S] and total sulfides [$\text{H}_2\text{S} + \text{HS}^-$] reached respectively $472.14 \pm 66.64 \mu\text{M}$ ($8391 \pm 2866 \mu\text{M}$) and $1378.79 \pm 185.53 \mu\text{M}$ (Fig. 1B-red and green lines) demonstrating that the anaerobic sulfate-reducing bacteria (SBR) contained into the bones were functional and produced sulfides by degradation of the organic matter thanks to sulfate reduction. At the same time, oxygen decreases quickly, from $1150.24 \mu\text{M}$ to almost $0 \mu\text{M}$, indicating that the veil is mostly anoxic (Fig. 1B-blue line). Thus, *Thiovulum* cells have to migrate from the surface (oxygen-rich area) to the bottom (sulfur-rich area) of the veil using O_2 and HS^- accordingly to their metabolic needs following a continuous slow movement (data not shown).

Ultrastructural and elemental analyses

The organic matters deployed close to *Rhizophora* roots in marine mangrove environment, were surrounded by a large veil of colorless microbial mat (Figs. 2A-B). These white veils were characterized by a patchy aspect (Fig. 2A), in which each dot was composed by slowly rotational motile, colorless, large spherical cells (Fig. 2C, cjm-2021-0101.R1suppla). The remaining of the veils contained *Thiovulum* sp. strain karukerense previously described from this environment (Gros 2017). Bright-field microscopy revealed the presence of large refractive granules (Figs. 2C-D) randomly distributed inside these spherical cells with a mean diameter of $47.75 \pm 3.56 \mu\text{m}$ (Figs. 2C-D).

According to SEM observations, these free-living bacteria appeared as single ovoid cells (Fig. 3A) with numerous flagella covering the cell surface (Fig. 3C). Most of the cells presented an invagination (Fig. 3B) on one side, such structure was also detectable using light microscope at high magnifications (data not shown). Internal granules close to the membrane cell are visible even in SEM (Figs. 3A, D). STEM observations showed that these bacterial cells had a double membrane typical of that in Gram-negative bacteria. No gas vesicles were detected. The “large empty vesicles” observed within the cytoplasm probably correspond to sulfur inclusions lost during the dehydration and embedding processes (Figs. 3E-F). To confirm the presence of sulfur within these granules, no dehydrated samples were analyzed by using an environmental SEM coupled to an EDX detector. The main component obviously detected in addition to classical elements such as C, N, O, Na and Cl is Sulfur (Figs. 4A-B), confirming the presence of elemental sulfur granules within the bacterial cytoplasm. The elemental sulfur would represent an intermediate oxidation product of sulfides, stored as refractive internal globules easily detected by light microscopy (Figs. 4C-D). Both, sulfur map (Fig. 4G) and the corresponding mixmap image (Fig. 4H), confirm the presence of elemental sulfur within all cells analyzed and allows us to position this species as a colorless sulfur-oxidizing bacterium.

Photosynthetic pigment analysis

Figure 5 shows the spectra obtained on a green leaf from *Rhizophora mangle* (red curve), a cyanobacterium colonizing the periphyton as *Oscillatoria* sp. (blue curve) and on *Candidatus* Thiovulum sp. strain imperiosus (green curve). The raman spectra of sea water and elemental sulfur (black and purple curves respectively) are also presented as references. The raman spectra obtained on the green leaf reveal several bands. The intensive bands visible at 1610, 1570 and 1496 cm^{-1} are characteristic of chlorophyll a (Cai et al. 2002). The raman bands detected from living cyanobacteria at 1520, 1150 and 1000 cm^{-1} are characteristic of carotenoids (Vitek et al 2020). The raman spectra obtained from living *Candidatus*

Thiovulum sp. strain imperiosus cells reveal a single intensive band at 450 cm⁻¹. We assumed that this band can be attributed to sulfur (Himmel et al. 2009) which is confirmed by the elemental sulfur spectra. These results allow us to conclude that none of main photosynthetic pigments encountered in mangrove ecosystems such as carotenoids and/or chlorophyll are present in the epsilon proteobacteraeota studied here.

Phylogenetic analysis of the large colorless bacterium

The phylogenetic reconstruction based on partial sequences (915pb) of the gene encoding for 16S rRNA, placed this organism within the cluster of *Thiovulum* (Fig. 6). The strain analyzed here exhibited the strongest homology (88.9% identity for a recovery of 98%) with *Thiovulum* sp. strain karukerense (KX641474) previously described from the same mangrove environment (Gros 2017), and various sequences recovered from epsilon-proteobacteraeota of the genera *Sulfurimonas*, *Sulfuricurvum*, *Sulfurovum*, *Sulfurospirillum*, and *Helicobacter* (between 83% and 87.1%) (Campbell et al. 2006; Handley et al. 2014). The phylogenetic reconstruction confirmed these affiliations (Fig. 6). This strain of marine mangrove colorless bacteria revealed quite weak homologies (88 to 88.9%) with other *Thiovulum* strains already described (*Thiovulum* sp. ES, *Thiovulum* sp. [AH003133], and *Thiovulum* sp. strain karukerense) for the over-lapping region concerned. This suggest that, while belonging to a same cluster, this new marine mangrove strain, *Candidatus* *Thiovulum* sp. strain imperiosus, could represent a new genus of *Thiovulum*.

DISCUSSION

Marine environments of fringe mangrove forests have been better investigated during this last decade. However, microbial communities of terrestrial mangrove soils and sediments (Rigonato et al. 2012, 2013; Varon-Lopez et al. 2014) are more studied than shallow-water marine mangrove microbial

mats that exist close to the roots of *Rhizophora mangle* trees. Upper part of these bacterial mats is usually constituted by filamentous cyanobacteria (Guidi-Rontani et al. 2014; Duperron et al. 2020), whereas their inner layers are composed of anoxygenic phototrophic bacteria (Lopez-Cortes 1990; Sheridan 1991; Gros et al. 2018). In coastal areas characterized by weak hydrodynamic conditions, large white patches of colorless sulfur-oxidizing bacteria covering the surface of the marine sediment can be observed as dense mats of gamma proteobacteria Beggiatoaceae (Jørgensen 1977; Jean et al. 2015) or as fragile veils of epsilon-proteobacteria (Gros 2017). Colonization of organic matter such as plant debris or bones by micro-organisms in mangrove is a multiple-step process involving many bacteria using different metabolisms. At first, branches and bones are colonized by a community of heterotrophic bacteria that will degrade dissolved organic compounds as well as particulate materials. These heterotrophic activities create anoxic niches that will be colonized by Sulfate Reducing Bacteria (SRB) allowing the production of sulfides (Smith and Baco 2003; Laurent et al. 2013). As described above, the sulfide production from wood and bone materials is clearly a result of organic matter degradation by anaerobic bacteria. Due to the high activity of SRB in the anoxic part of the marine sediment, mangrove can produce high level of sulfides reaching up to 10mM (Crémière et al. 2017) sustaining the development of a large variety of sulfur-oxidizing bacteria as free-living organisms (Jean et al. 2015; Gros 2017) or as symbionts of various marine hosts (Frenkiel et al. 1996; Schlegel and Jannasch 2006; Dubilier et al. 2008; Himmel et al. 2009; Laurent et al. 2013; Abouna et al. 2015). Thioautotrophic proteobacteria (whatever the class concerned) can be observed on the sediment (Jean et al. 2015; Gros 2017) and also on decaying remains of dead animals, seaweed tissue, plants and decaying organic matter producing sulfides due to a SRB activity (Fauré-Fremiet and Rouiller 1958; Jørgensen 1977; Fenchel and Bernard 1995; Laurent et al. 2009, 2013; Smith et al. 2015). Veils of *Candidatus* Thiovulum sp. strain imperiosus covering the sediment are rather rare and difficult to observe in the field contrary to *Candidatus* Thiovulum sp. strain karukerense veils (Gros 2017). On the other hands, *Candidatus* Thiovulum sp. strain imperiosus veils are easy to obtain and

observe in experimental condition and *in situ*, on decaying substrates (preferentially bones). The two veils are easily distinguishable as *Candidatus Thiovulum* sp. strain imperiosus forms a veil with dots distinguishable with eyes during snorkeling. Although several species of *Thiovulum* can be found in the same veil, it is more common to see veils composed of a single species on the surface of mangrove sediments and organic substrates. Each veil has its own specific patterns that are easily recognizable according to the majority species that composes it.

Among the sulfur-oxidizing bacteria, *Thiovulum* spp. cells were previously described as round to ovoid and measure 5 to 25 μm in diameter (Jørgensen 1977; Robertson et al. 2015). On the contrary, *Candidatus Thiovulum* sp. strain imperiosus, are single ovoid cells with a mean diameter of 47.75 ± 3.56 μm , about 3.5 times larger than the previous *Thiovulum* strain characterized from the same environment (Gros 2017). According to bright-field microscopy, *Candidatus Thiovulum* sp. strain imperiosus cells present a rotational pattern of motility (they turned on themselves). Their path and speed of swimming motility within the optimal H_2S - O_2 gradients zone in veil is probably modulated by (i) the availability of substrate as already described for *Thiovulum majus* (Fenchel and Glud 1998; Taylor and Zhulin 1998), and by (ii) the number of flagella available compared to the size of the bacterial cells.

When growing at the oxic/anoxic interface (in presence of both H_2S and O_2), many sulfur-oxidizers store transiently, or not, sulfur granules inside the cells (Wirsén and Jannasch 1978; Pickering et al. 2001; Thar and Fenchel 2001; Dahl and Prange 2006; Laurent et al. 2009). The presence of highly refractive and anisotropic intracellular granules gives the *Thiovulum* cells a white appearance in incident light and attests to their crystalline texture (Fauré-Fremiet and Rouiller 1958). Size of the sulfur granules present in such cells is larger than those (100 nm to over 1 μm in diameter) described by Shively (1974). The presence of elemental sulfur detected by EDX indicated that the bacteria may have used internal storage material such as S^0 as substrate and energy to face chemical variations in their environment (Jørgensen and Revsbech 1983; Schmidt et al. 1987; Wirsén et al. 2002). This result was consistent with those previously

obtained on sulfur-oxidizers belonging to the genus *Chromatium* (Sun et al. 2017) and more recently on *Candidatus Thiovulum* sp. strain *karukerense* cells (Gros 2017).

Thiovulum strains have been isolated from the production water of an oil well (Gevertz et al. 2000), and several 16S rDNA sequences have been obtained from hydrothermal vents (Moyer et al. 1995; Corre et al. 2001), marine sediments (Madrid et al. 2001), and cave springs (Engel et al. 2003). While several morphotypes have been described, only a few sequences are available from the database for phylogenetic analysis. In this study, as the bacteria cells can be easily and individually sorted under a light microscope, we decided to directly sequence the PCR products obtained. The main objective here was not to produce an overview of the bacterial population present in such veils by metabarcoding analysis but to focus only on these large bacteria easy to individually collect in the laboratory. The closest sequence (88.9% identity for a recovery of 98%) to this new large *Thiovulum* strain described from mangrove is *Candidatus Thiovulum* sp. strain *karukerense* (KX641474). These distances suggest that these strains, defined as "*Thiovulum*", may actually belong to at least two different species, even to two different genera (Yarza et al. 2014). However, a larger number of longer 16S rDNA sequences are required to determine the phylogeny of "*Thiovulum*" accurately. Thus, the strain collected from the marine mangrove environment may correspond to a new species within this poorly studied genus of epsilon-proteobacteria. The deepening of knowledge concerning this specific large new epsilon-sulfur-oxidizing *Thiovulum* strain and its lifestyle to marine mangrove ecosystem, as well as its contribution to the diversity of *Thiovulum* communities and to the microbial communities of marine mangrove will be a part of further metagenomic and single-cell sequencing studies.

Ex situ measurements done in aquaria with immersed bones brought back to the laboratory 4-5 days after deployment, showed that the introduction of electrodes has not disturbed the veil structure developed around the bones. This may be due to the organization of *Thiovulum* communities in the bacterial veil covering the surfaces of marine sulfide deposits, in which cells are either free-swimming cells

or cells attached to the bone surface or to neighboring cells include in a matrix constituted by a 10-100 μm long mucous stalks (Wirsén and Jannasch 1978; Fenchel 1994; Schaudinn et al. 2007; Petroff et al. 2015). During the experiments presented here, the water column was normally oxygenated but oxygen decreases quickly to reach zero inside the veil. These results indicate that veils are mostly anoxic and enclose anoxic water by limiting molecular diffusion (Peulen and Wilkinson 2011) and creating a micro-environment around the bones in which sulfides accumulate and oxygen is depleted. The data presented here from *ex situ* measurements confirm those of Treude et al. (2009), who measured a maximum sulfide concentration of 3.7 mM at the sediment surface surrounding a whale carcass and oxygen below detection limit, while in surrounding sea water oxygen concentration was around 260 μM and sulfides were not detected. One explanation would be that the anaerobic degradation of bone lipids by SRB produce enough sulfides to support these sulfur-oxidizing-bacterial veils (Smith et al. 1989; Watanabe et al. 2000).

A survey of the development of microscopic organisms during the deployment of the bones showed a clear temporal dynamics of bone colonization revealing that the succession of sulfur-oxidizers related with the rapid evolution of sulfide concentration on the bone surface. Sulfide was detected *in situ* on the surface of the deployed bones using similar autonomous sulfide electrodes previously used for wood fall surveys (Laurent et al. 2009, 2013). Up to 1 mM of sulfide was detected only 36 h after deployment and for a duration of 10 days (Gros, unpublished data). The rapid development of *Thiovulum* veils (2 days after the bone immersion) is consistent with its strict dependence on sulfides, so just after the establishment of real sulfidic conditions on the substrates. By comparison with the reference model of whale falls (Smith and Baco 2003; Smith et al. 2015), even if the survey presented here was done on a much shorter timescale (days versus months or years), no marked mobile-scavenger stage (ex: Hagfish, clams, gastropods, shrimp, polychaetes, crustaceans, *Osedax* worms and limpets) has been identified as we have cleaned the bones before immersion. On the other hand, a strict sulfophilic stage (as defined

from the predominance of thioautotrophic bacteria) was observed on the bones within only 2 days after deployment.

Pattern bones colonization, showed a succession of several bacterial species after the production of sulfides from the bones. *Thiovulum* sp. strain *karukerense* cells always appeared first after 2 days of deployment, followed by protists belonging to the genus *Trochochilodon* as bacterivorous predators. Then, this first population of *Thiovulum* species is replaced by *Candidatus* *Thiovulum* sp. strain *imperiosus* as dominant organism 5 to 7 days after deployment. Finally, these ϵ -proteobacteriaeota are decimated 10 days after the deployment by the arrival of dense populations of bacterivorous ciliates concomitant to the decrease of sulfides (Grimonprez, personal observation). This study showed that sunken bones in tropical shallow water followed a different colonizing scheme than proposed by Laurent et al. (2013) for wood falls (*Rhizophora mangle* and *Cocos nucifera*) immersed for 3 months. Indeed, these authors showed a later colonization than for the bones and different microbial populations: few organisms at the wood surface (some heterotrich ciliates, gastropod) after 4 days of immersion, and an increase in the diversity of wood colonizers after 10 days (annelids, sedentary polychaetes, crustaceans, other ciliates, nematodes, some ascidians and ectoprocts). Colonial *Zoothamnium niveum* and *Pseudovorticella* sp. were abundant. However, the presence of large colorless bacteria was not observed on these substrates. This difference in colonization time between the two supports could be explained by the intrinsic nature of these organic substrates and their accessibility bacteria. Indeed, we can note that the sulfide production from anaerobic bacterial decomposition of bone lipids is faster (first sulfides emitted in the first 10 hours, Gros. personal observations) than this from wood falls in mangrove (4 days for *Rhizophora mangle* and 6 days for *Cocos nucifera* [Laurent et al. 2013; Grimonprez et al. 2018]) and from whale falls in deep sea (Treude et al. 2009; Danise et al. 2012), but the sulfides remain present longer in the wood (more than six months). Furthermore, the sulfide concentrations detected in this study from sunken bones are higher than those detected in woody debris (300 μ M detected on pieces of pine wood placed 13 weeks in aquarium (Yücel

et al. 2013) and up to 114 μM on sunken woods in mangrove environments (Laurent et al. 2009). These data suggest that bone lipids would be a faster source of sulfide release than woody substrates and a more readily available source of sulfur for epsilon-proteobacteria.

The contribution of epsilon-proteobacteria (and by extension to all thioautotrophic bacteria) to C and N cycles and their role in sustaining local food webs in marine mangrove environments are under investigation. The real contribution of *Thiovulum* communities to total primary production in marine mangroves as well as their involvement as prey in the food web is complex and requires further studies for clarification.

Nucleotide sequence accession number

Candidatus Thiovulum sp. strain imperiosus 16S rDNA partial sequence obtained in this study was deposited in the GenBank database under accession number MH714858.

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FIGURE CAPTIONS

Figure 1. Environmental Oxygen and Sulfide measurements in *Thiovulum* veil.

A: Bones were collected after 3 days of deployment, delicately placed with their surrounding water and veil in an oxygenated aquarium in the laboratory. Oxygen (Unisense OX-100) and hydrogen sulfide (Unisense H₂S-100) microsensors probes are respectively positioned in the 500 to 1100 µm of water surrounding the oxic/anoxic interface and inside the veil. The distances of the microsensors probes from the oxic/anoxic interface were measured with a micromanipulator (MM33-unisens).

B: Concentrations profiles (µM) of oxygen (blue curve) and hydrogen sulfide (H₂S: red curve, total sulfide Σ (H₂S+HS⁻): green curve) are measured 5 days after deployment, continuously during 90 minutes. Time interval between two measurements is one minute. The dotted black arrow indicates the veil interface between the oxic zone (left) and the anoxic zone (right).

Figure 2. Description of white veil of “*Candidatus Thiovulum* sp. strain imperiosus” covering deployed bones in marine mangrove.

A: *Thiovulum* cells appear as white patches on the bone surface (arrow) constituting a continuous translucent and whitish *Thiovulum* veil (asterisks) after 5-7 days of deployment at 1.30 m depth and about 20-30 cm above the bottom of sediment using a metallic holder.

B: Light micrograph, of freshly sorted individuals of *Candidatus Thiovulum* sp. strain imperiosus. The main characteristics are the white color of the cells and their spherical shape with a granular aspect.

C and D: Structural analysis of *Thiovulum*. Light microscopy of the freshly collected cells observed and photographed without staining and dehydration (C) with Nomarski Axioskop2 contrast (D). This large bacterial cell shows a spherical structure delimited by a cell wall (red head arrow), about 45-50 µm of diameter, with many transparent and dense cytoplasmic granules with white dots characteristics of

elemental sulfur crystals refracting the incident light in a wide range of colors (white arrow). Some cells seem to be invaginated (red arrows).

Figure 3. Ultrastructural analysis of *Thiovulum* cell.

A : SEM view of *Thiovulum* cell characterized by a spherical shape with a total diameter of 49 μm . Elemental sulfur granules (SG) situated below the membrane cell are distinguishable.

B: *Candidatus* *Thiovulum* imperiosus (black star) sometimes coexists in the same veil with individuals of *Candidatus* *Thiovulum* karukerense (white stars) which are easily differentiated by their smaller diameter (12-15 μm) compared to the giant size of individuals of *Candidatus* *Thiovulum* imperiosus (45-50 μm).

C: Numerous flagella are visible on the entire surface of the bacteria and explain the mobility of *Thiovulum* cells that move in turning on themselves.

D: Higher magnification focusing on the numerous cytoplasmic sulfur granules in contact with the cell wall as seen from light pictures (figures 2C-D).

E and F: TEM microphotographs of *Thiovulum* cell confirm its spherical shape with a clear invagination (white arrow head). No nucleus, organelles and/or symbionts were detected inside the cells confirming its status of free-living bacterial strain.

Large transparent areas within the cell represent sulfur vesicles (V) which appear empty due to the loss of sulfur during acetone dehydration process. Such vesicles appear to be encased by a unit membrane. The cell wall of this bacterial strain is indicated by dotted arrows and the flagella outside the bacteria by straight arrows. The cytoplasm of the cells appears finely granular.

Figure 4. Sulfide metabolism of *Thiovulum*.

A: ESEM image of a *Thiovulum* cell freshly fixed 2 hours at 4°C in 2.5% glutaraldehyde in 0.2 μm filtered seawater. This view suggests the existence of sulfur granules (white dots) within the bacterial cytoplasm.

B: EDX spectrum of *Thiovulum* cells obtained from non-dehydrated samples observed under an ESEM shows a peak of elemental sulfur, confirming that *Thiovulum* contain sulfur as main component of the cells. This suggests that *Candidatus Thiovulum* sp. strain imperiosus is a sulfur oxidizing bacteria (C: carbon, O: oxygen, Na: sodium, S: elemental sulfur, Cl: chloride).

C to H: EDXS mapping of Carbon (C: blue), Oxygen (D: green), Sodium (E: yellow) and Chloride (F: grey) show that these elements are distributed uniformly over the surface of the bacteria, so they are not specific. Conversely, the sulfur mapping localizes elemental sulfur (G: marked in red) inside the cells within the cytoplasmic granules. A mixed image (H) demonstrates the superposition of sulfur (G) on the intracellular granules (A: white dots).

Figure 5: Analysis of *Candidatus Thiovulum* sp. strain imperiosus by Raman microspectrometry.

Raman spectra acquired from individual cells confirm that none of main photosynthetic pigments encountered in mangrove ecosystems either from plant (as chlorophyll a, red line) or from bacteria (as carotenoids, blue line). Only elemental sulfur was detected (purple line) confirming that this large colorless bacterium contains sulfur granules in its cytoplasm. The black line shows that the surrounding seawater (used as negative control) remain free of sulfur and photosynthetic pigments.

Figure 6. Maximum likelihood phylogenetic tree based on 16S rRNA gene sequences (842 bp) within related species of the epsilon proteobacteraeota class.

The phylogenetic tree was constructed using the Clustal X (Thompson et al. 1997) and MEGA version 7 (Kumar et al. 2016) programs. Gaps in sequence alignments were omitted in the calculation. The sequence accession numbers are within parentheses. The bootstrap values obtained by 1000 replications (Felsenstein 1981, 1985) over 50 % are beside nodes. The outgroup was specified as *Agrobacterium tumefaciens* (D14500). Bar, 5 nucleotide substitutions per 100 nucleotides.

Figure 1.

Figure 2.

Figure 3.

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Figure 4.

Figure 5.

Figure 6.