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SHORT NOTES

Localization of endobacteria in the gastrodermis of a Mediterranean gorgonian coral, *Paramuricea clavata*, using fluorescence in situ hybridization

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Abstract Gorgonian corals are among the most representative species of hard-bottom sublittoral ecosystems in the Mediterranean Sea. The red gorgonian, *Paramuricea clavata*, harbors a specific, spatially and temporally stable bacterial community that may have an important biological role in the function of the holobiont. However, little is known about the microbial–gorgonian relationship, specifically the nature of the interaction established by the bacteria with their host. Documenting the distribution of microorganisms within coral tissue compartments is critical for providing an overall framework for understanding their functional roles. Here, we used histology and fluorescence in situ hybridization (FISH) to detect and localize bacteria in the tissues of *P. clavata*. Aggregates of tightly packed bacteria were found in the endoderm of the polyps, which is consistent with previous observations of endobacteria in the tissues of scleractinian coral species. This finding suggests the existence of an intimate biological integration between specific members of the microbiota and gorgonian corals that likely results from a complex interplay mediating recognition and maintenance of the bacterial associates. In addition to providing the basis for the development of FISH studies in gorgonians, this work includes the first histological description of the inner structure of *P. clavata* polyps, illustrating the typical features of general gorgonian anatomy.

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Introduction

Corals are associated with diverse assemblages of bacteria that are believed to contribute to their overall health and adaptive response to environmental changes (Rosenberg et al. 2007; Bourne et al. 2009). Among other putative functions, coral-associated bacterial communities likely play substantial roles in supporting the host's nutrient budget and defense against pathogens (for reviews, see Mouchka et al. 2010; Krediet et al. 2013). Despite growing evidence of a critical contribution of bacterial interactions to the healthy functioning of the coral holobiont, relatively little is known regarding the nature of these associations and the spatial distribution of the microbiota within the coral host. Therefore, investigating the architecture of bacterial communities is essential to characterize the microbial processes performed by the natural assemblages in situ and to understand the mechanisms underlying their maintenance within the coral niche. Given increasing stress factors and the worldwide decline of coral health (Hughes et al. 2010), such information could also yield valuable insights into the conditions that may cause the destabilization and breakdown of the holobiont.

In the Mediterranean Sea, gorgonian corals (*Cnidarian*, *Octocorallia*) are important structural organisms in coralligenous outcrops, which are one of the most diverse coastal ecosystems (Ballesteros 2006). Among the emblematic long-lived species typically found in coralligenous assemblages is the red gorgonian, *Paramuricea clavata*,

a temperate, azooxanthellate octocoral that forms dense populations in dim-light habitats. Culture-independent surveys of the composition and dynamics of the bacterial communities associated with *P. clavata* have revealed the presence of host-specific bacterial associates that are conserved throughout seasons and among different geographic locations. These surveys provided a first insight into the microbiology of this species (La Rivière et al. 2013). Recent phylogenetic analyses have also indicated that the dominant bacterial taxa associated with *P. clavata* and several other gorgonian species may coevolve with their host, suggesting an intimate partnership within the gorgonian holobiont (La Rivière et al. 2015). In addition to genetic-based analyses, further research, such as the study of the spatial arrangement of bacteria within gorgonian tissues, is needed to delineate their potential role and the basis of host specificity.

Fluorescence in situ hybridization (FISH) allows direct observation and identification of bacterial cells in complex natural samples without prior cultivation (Amann et al. 1990b; Amann and Fuchs 2008). This technique has been successfully applied to visualize bacteria associated with different marine invertebrates, such as sponges (Webster et al. 2001), bivalves (Jensen et al. 2010) or crustaceans (Meziti et al. 2010). However, relatively few studies have investigated the localization of individual bacterial cells in corals, notably because coral tissues exhibit a strong endogenous fluorescence that hinders their detection by FISH (Salih et al. 2000; Dove et al. 2001). In certain instances, this problem has been circumvented through improvement in the FISH probe–fluorochrome detection step (e.g., Ainsworth et al. 2006). In particular, Neulinger et al. (2009) applied a catalyzed reporter deposition (CARD)–FISH technique that provides a substantial increase in fluorescence intensity and allows more sensitive detection of the hybridized bacteria in coral tissues (Bobrow et al. 1989; Pernthaler et al. 2002). Therefore, the improvement in direct imaging techniques in future studies will reveal increasing details of the spatial arrangement of coral-associated bacteria.

In this study, our specific objectives were to detect and localize the bacterial associates in the tissues of *P. clavata* through the adaptation of a CARD–FISH protocol. Because there is currently limited information regarding the internal anatomy of this species, we also aimed to describe the gross organization of the polyp tissues using a histological analysis of stained sections. To the best of our knowledge, this is the first report to provide structural observations of this gorgonian species. The present study will help lay the foundation for future detailed analyses of the nature and significance of the microbial interactions in a temperate gorgonian holobiont.

Materials and methods

Sampling, fixation and decalcification

Apical fragments (2 cm in length) of *P. clavata* were sampled from three colonies at the Riou site (France; 43°10.345′N, 05°23.319′E) in May 2012 as previously described (La Rivière et al. 2013). The samples were soaked in three washes of sterile-filtered seawater to remove loosely attached bacteria and left in a final bath for 4 h at 15 °C to allow polyp extension. Menthol crystals were added to anesthetize the polyps during a further incubation of 2 h at 4 °C (Lonkar et al. 2011) prior to fixation of the samples in 4 % formalin for 16 h and decalcification in 20 % (wt/vol) EDTA with PBS (pH 7.4) for 48 h at 4 °C. Fragments were subsequently transferred to a 50 % (vol/vol) ethanol–PBS solution (pH 7.4) and stored at −20 °C until use.

Thin sectioning

The fixed tissues were sequentially processed through 70, 80 and 95 % ethanol followed by three 100 % ethanol and three xylene washes, for 1 h each, prior to paraffin embedding. Tissue sections (5–10 µm) were deparaffinized by washing in two baths of Neo-Clear (Merck) for 15 min each and rehydrated in a graded ethanol–water series (twice in 100 % ethanol, once in 95, 90 and 80 % [vol/vol]); they were washed twice in sterile ultra-pure water for 5 min each and then air-dried.

For the light microscopy observations, the sections were stained with a standard hematoxylin and eosin protocol (Sigma-Aldrich) or a Gram staining kit (Sigma-Aldrich) according to the manufacturer's instructions. For fluorescence in situ hybridization, the slides were processed as described below.

Permeabilization

Bacterial cell walls were permeabilized by incubating the slides in 80 µl of lysozyme solution (1355.10^6 U of lysozyme [Sigma-Aldrich] ml^{-1}, 500 mM EDTA [pH 8.0], 300 mM Tris–HCl [pH 8.0]) at 37 °C for 2 h in a humidified chamber. After three 1-min washes with sterile ultra-pure H_20 at room temperature, the samples were permeabilized with 80 µl of achromopeptidase buffer (60 U of achromopeptidase [Sigma-Aldrich] ml^{-1}, 10 mM NaCl, 10 mM Tris–HCl [pH 8.0]) at 37 °C for 1 h. Subsequently, the slides were washed with sterile ultra-pure H_20 and air-dried. Endogenous peroxidases were inactivated by incubating the thin sections in 80 µl of 10 mM HCl solution for 30 min, followed by three washes with sterile H_20.

Autofluorescence reduction

The permeabilized slides were buffered in PBS (pH 7.4) for 3 min, followed by 3 min in 70 % ethanol. The thin sections were then covered with Autofluorescence Eliminator Reagent (Chemicon–Millipore) and placed in a humidified chamber overnight (14–16 h). After incubation, the slides were washed three times in 70 % ethanol to ensure complete elimination of the dark reagent, air-dried and stored at −20 °C before hybridization.

Hybridization

Four horseradish peroxidase-labeled oligonucleotide probes were used: the universal eubacterial probes EUB338, EUB338-II and EUB338-III in an equimolar mix (Amann et al. 1990a; Daims et al. 1999) and the control probe non-EUB338 complementary to EUB338 (Wallner et al. 1993). The thin sections were covered with a hybridization solution containing 1 ng μl^{-1} of probe in hybridization buffer. The hybridization buffer consisted of 55 % formamide, 900 mM NaCl, 20 mM Tris–HCl (pH 8.0), 0.05 % Triton X100 (Sigma-Aldrich), 10 % (wt/vol) dextran sulfate (Sigma-Aldrich) and 10 % blocking solution. The blocking solution (pH 7.5) consisted of 10 % (wt/vol) Blocking Reagent (Boehringer Mannheim), 100 mM maleic acid (Sigma-Aldrich) and 150 mM NaCl. The hybridization was performed at 35 °C for 4 h in a humid chamber. Thereafter, the sections were transferred to 50 ml of prewarmed washing buffer (13 mM NaCl, 20 mM Tris–HCl [pH 8.0], 5 mM EDTA [pH 8.0] and 0.01 % [wt/vol] sodium dodecyl sulfate) at 37 °C for 15 min. The slides were soaked for a few seconds in cold sterile ultra-pure water and quickly air-dried.

Amplification

The thin sections were equilibrated with PBS (pH 7.4) for 15 min in the dark. The tissues were covered with a 1:100 (vol/vol) mixture of tyramide-Alexa488 and amplification buffer (10 % [wt/vol] dextran sulfate [Sigma-Aldrich], 2 M NaCl, 10 % blocking solution, 0.0015 % H_20_2 and PBS [pH 7.4]) and incubated at 46 °C for 30 min in a dark humidified chamber. After amplification, the slides were washed in PBS (pH 7.4) for 20 min at room temperature and three times for 1 min each in sterile ultra-pure water. The sections were air-dried and covered with a DAPI mix mounting medium that allowed counterstaining of the cells (1 µg ml^{-1} DAPI [4′,6′-diamidino-2-phenylindole], 77 % [vol/vol] Citifluor AF1 [Citifluor Ltd], 14 % [vol/vol] Vectashield mounting medium [Vector Laboratories]).

Microscopy and image processing

The hybridized thin sections were viewed using a Zeiss LSM 780 confocal microscope. The fluorescence was excited sequentially with the 405 and 488 nm laser lines, and the emission was detected in the range from 415 to 469 nm for DAPI and from 497 to 552 nm for Alexa488. Image stacks (steps of 0.5–1 µm) were acquired with GaAsP photomultiplier tube (PMT) detectors. The 32 channels GaAsP PMT detectors allowed spectral detection in parallel mode in the range from 412 to 692 nm. Composite and overlay images were produced from these stacks with ImageJ, v1.46 software (http://imagej.nih.gov/ij).

Results and discussion

Because the histological descriptions of gorgonian corals are scarce, a preliminary analysis of the *P. clavata* anatomy was needed as a prerequisite for the in situ localization of bacteria. Microscopic examination of hematoxylin–eosin-stained sections demonstrated that the inner structure of *P. clavata* was in agreement with the previously described anatomy of gorgonians (Galloway et al. 2007). All the examined polyps from the sampled colonies were retracted or moderately expanded, indicating an incomplete effect of the anesthesia procedure. A longitudinal section of polyps retracted in their calyx is shown in Fig. 1a. The central axis consisted of a wide, apparently hollow central core surrounded by concentric layers of proteinaceous gorgonin, which is consistent with the morphology described for other members of the Plexauridae family (Bayer 1981; Lewis et al. 1992). Gonads of different sizes (approximately 100-300 µm in diameter) were found in the polyp cavity of the analyzed sections. In the transverse sections, the pinnate tentacles surrounding the mouth were clearly observed (Fig. 1b). Within the mesoglea, a large number of openings were visible corresponding to the lacunae that remained after decalcification of the sclerites embedded in the host tissue (Nonaka et al. 2012). The mesenteries dividing the gastric cavity could also be discerned (Fig. 1b). Therefore, we conclude that the structural integrity of *P. clavata* tissues, which was required for in situ localization of bacterial associates, was maintained throughout the steps of our histological protocol.

In a first attempt to determine the presence of bacteria within the tissues of *P. clavata*, we examined Gram-stained sections of polyps using light microscopy. Putative bacterial aggregates with a round to ovoid shape were consistently observed within the gastrodermal tissue lining the gastric cavity (Fig. 1c, d). The aggregates stained Gram-negative, and their sizes ranged from approximately 10 to 25 µm in maximum diameter. Similar bacterial aggregates

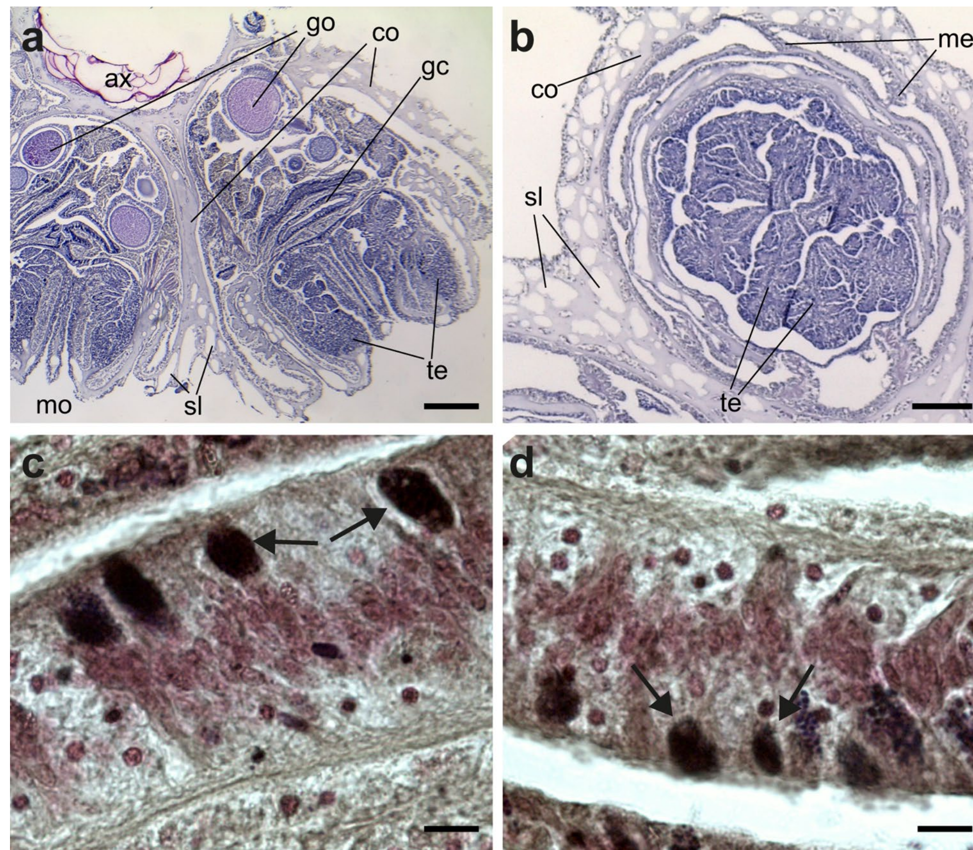

Fig. 1 Histological sections of *P. clavata* polyps. Longitudinal (**a**) and transversal (**b**) sections of polyps stained with hematoxylin and eosin. *ax* axis, *co* coenenchyme, *gc* gastric cavity, *go* gonad, *me* mesentery, *mo* mouth opening, *sl* sclerite lacunae, *te* tentacle. **c** and **d** Putative bacterial aggregates (*arrowheads*) within the gastrodermal tissue layer of Gram-stained sections. *Scale bars*: 300 µm (**a**), 500 µm (**b**), and 10 µm (**c** and **d**)

were observed in the tissue of several species of scleractinian corals using histology and light microscopy (Sudek et al. 2012; Work and Aeby 2014). Notably, microbial aggregates were also found in the tissues of sea anemones (McKinstry et al. 1989; Schuett et al. 2007), indicating that such intimate bacterial–host associations may be a general feature of cnidarians. In the *P. clavata* sections, bacteria-like forms could be discerned within certain aggregates and appeared as densely packed spheres of approximately 1–2 µm in diameter. Additional research using electron microscopy is necessary to describe the morphology of the bacteria and their arrangement within the aggregates. Using the Gram stain, no bacteria were detected in other portions of the polyps, such as in the gonads or in the peripheral surface tissues of the gorgonian corals.

Previous attempts to visualize the coral-associated bacterial communities using the FISH procedure have been limited by the endogenous fluorescence of coral tissues (Salih et al. 2000; Ainsworth et al. 2006). A preliminary analysis of *P. clavata* sections also revealed a high level of autofluorescence that precluded detection of the specific labeling probe (not shown). To overcome this limitation, we resorted to a tyramide-based amplification reaction (CARD-FISH) designed to increase the signal intensities of hybridized bacteria (Bobrow et al. 1989; Pernthaler et al. 2002; Neulinger et al. 2009) and improved the protocol by incubating the tissue sections with an autofluorescence eliminator reagent before the in situ hybridization. This method resulted in a substantial decrease in the background autofluorescence of gorgonian tissues for a range of wavelengths that corresponded to the emission of the FISH probe fluorochromes (Fig. 2).

The *P. clavata* tissue sections were hybridized with a combination of EUB338 probes that target the majority of the Bacteria domain (Daims et al. 1999). Bright fluorescence signals were observed in the endodermal tissues surrounding the gastric cavity and within the proximal regions of the tentacles (Fig. 3a). The fluorescent bacteria were packed in ovoid aggregates of approximately 12-20 µm in length. Based on their size and shape, these aggregates resembled the Gram-stained bodies within the gastrodermal tissues of *P. clavata* observed under light microscopy

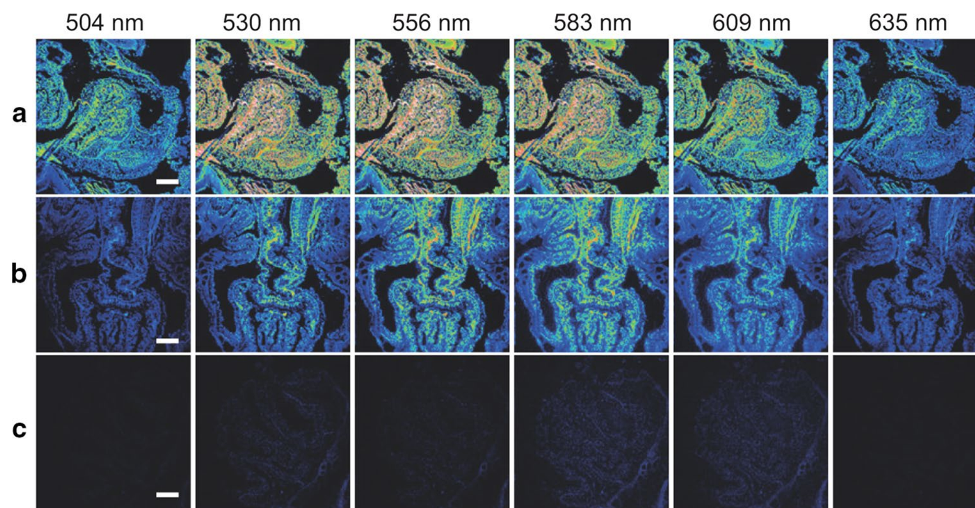

Fig. 2 Multispectral imaging of tissue autofluorescence. Confocal micrographs of *P. clavata* sections that are untreated (*lane* **a**) and treated with autofluorescence eliminator reagent for 50 min (*lane* **b**) or 16 h (*lane* **c**). *Artificial colors* indicate different levels of autofluorescence (*black*: no fluorescence, *blue*: lower fluorescence yield, *yellow–orange*: higher autofluorescence) emitted at the imaging wavelengths (504–635 nm) following illumination at 405 nm. *Scale bars*: 100 µm

(Fig. 1c, d). However, our imaging technique was not sensitive enough to visualize single fluorescent bacterial cells, precluding the observation of their arrangement within the aggregates. The hybridized sections of *P. clavata* were also counterstained with DAPI to confirm that the structures detected upon hybridization with the EUB338 probes corresponded to DNA-containing cells (Fig. 3b). No other bacterial signals could be observed on the peripheral, outer surface of the cross-sectioned polyps, thus indicating that the gorgonian microbial community was primarily composed of endobacteria.

Previous studies using FISH documented bacterial aggregates associated with the gastrodermal tissue of several species of scleractinian corals (Ainsworth et al. 2006; Ainsworth and Hoegh-Guldberg 2009). In two coral species from the Great Barrier Reef, *Acropora aspera* and *Stylophora pistillata*, the bacterial aggregates within the gastrodermis consisted of *Gammaproteobacteria* (Ainsworth and Hoegh-Guldberg 2009). Further FISH analysis revealed that tissue-associated aggregates in *S. pistillata* are composed of members of the *Endozoicomonas* genus, within the *Oceanospirillales* order (Bayer et al. 2013b). Recently, *Endozoicomonas*-related ribosomal sequences have been isolated from an increasing number of healthy coral species (Hansson et al. 2009; Kvennefors et al. 2010; Morrow et al. 2012; Speck and Donachie 2012). Significantly, we have shown that the bacterial community associated with *P. clavata* is largely dominated by gammaproteobacterial sequences that belong to *Endozoicomonas*-related bacteria (La Rivière et al. 2013). *Endozoicomonas* have also been identified as dominant bacterial associates in two other Mediterranean gorgonians, *Eunicella cavolini* and *Eunicella singularis* (Bayer et al. 2013a; La Rivière et al. 2015). Thus, the fluorescent aggregates identified by CARD–FISH in *P. clavata* tissues most certainly correspond to the prevalent *Endozoicomonas* bacterial associates. However, additional FISH studies with group-specific probes are needed to assess their taxonomic identification and examine their presence within the tissues of other gorgonian species.

Despite accumulating evidence that natural coral-associated populations such as *Endozoicomonas* spp. contribute to the maintenance of overall coral health (Meyer et al. 2014; Lee et al. 2015; Tout et al. 2015b), their precise functional role is not known. Bacterium–animal endosymbiosis is often related to nutritional interactions with the eukaryotic partner and may be essential to complement the metabolic networks of the host (Moya et al. 2008). Bacteria belonging to the *Oceanospirillales* order enable heterotrophic degradation of various organic substrates by producing extracellular hydrolytic enzymes (Garrity et al. 2005). Notably, bone-eating marine worms of the genus *Osedax* harbor intracellular *Oceanospirillales* that might help their host exploit complex carbon sources such as collagen from whale bones (Goffredi et al. 2007). It is thus tempting to speculate that the localization of bacteria in the coral gastrodermis could be related to nutrient acquisition. Tropical corals and temperate gorgonians are suspension feeders that capture and ingest zooplanktonic prey such as copepods and nauplii (Coma et al. 1994). The production of degradative enzymes by the resident population of endobacteria could therefore aid the digestion of recalcitrant compounds originating from zooplankton and contribute to supply the host with essential nutrients that have been

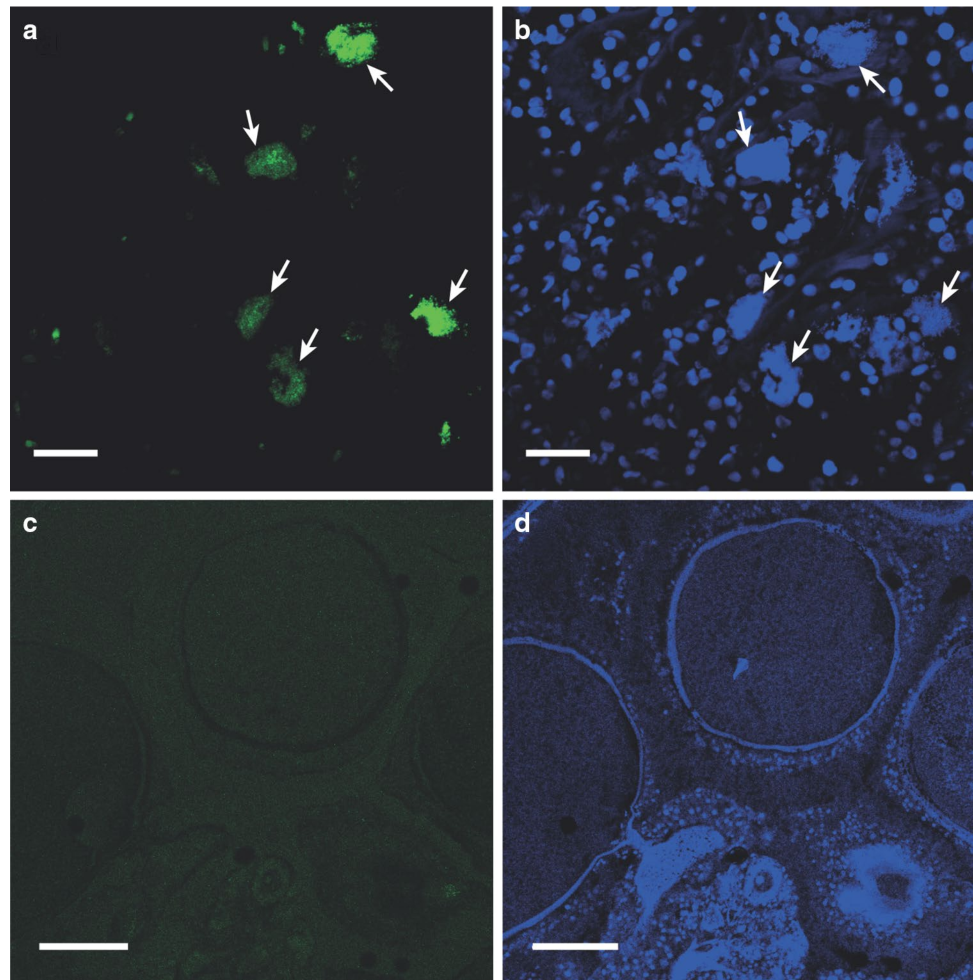

Fig. 3 Confocal micrographs of fluorescence signals in tissues of *P. clavata*. Hybridization signals of the universal bacterial probes EUB338 (**a**) and DAPI signals (**b**) within the endodermal tissues of a cross-sectioned polyp. Bacterial aggregates are indicated by *arrowheads*. EUB338 probes signals (**c**) and DAPI signals (**d**) in sections of oocytes. *Scale bars*: 20 µm (**a** and **b**), 100 µm (**c** and **d**)

reported to accumulate in the coral gastric fluid (Agostini et al. 2012). Nutrient availability is probably an important constraining factor for the ecology of azooxanthellate gorgonians, such as *P. clavata*, that cannot beneficiate metabolites from algal partners and are strongly dependent on feeding to sustain their metabolism (Coma et al. 1994). Because food availability is also a critical determinant of the survival of *P. clavata* during thermal stress (Coma et al. 2009), the possible trophic interaction between the endobacteria and their host may contribute to the physiological adaptation of gorgonians and their resilience to adverse environmental conditions. Potential beneficial roles of *Endozoicomonas*-related bacteria other than food digestion have been suggested in sulfur recycling (Raina et al. 2009), carbohydrate metabolism (Ding et al. 2016) and production of antibacterial compounds (Rua et al. 2014).

Based on histological slides, all sampled colonies produced female gonads, and no sperm sacs (male gonads; Coma et al. 1995) were observed. No fluorescent bacteria were detected in the oocytes of the examined polyps (Fig. 3c, d), which suggests that *P. clavata* does not transmit bacteria vertically via gametogenesis and spawning. Although further research (e.g., hybridization of male colonies) is required, this observation is consistent with previous studies showing that spawning corals acquire their bacterial associates via horizontal uptake during early developmental stages, after the release and fertilization of larvae (Apprill et al. 2009; Sharp et al. 2010). Assuming that *P. clavata* bacterial associates are acquired each generation from the surrounding environment, their establishment within tissues likely depends on complex processes involving selective attraction, recognition and entry steps (Bright and Bulgheresi 2010). Chemoattraction to various compounds released by the corals can induce free-living bacteria to move toward their host, resulting in physical contact between the two partners. Chemotaxis and motility

functions identified in coral-associated bacteria, including *Endozoicomonas* spp., might contribute to this behavior (Tout et al. 2015a). Some mechanism of recognition must exist to determine which bacteria can enter coral tissues. In scleractinian corals, the establishment of algal symbionts within the gastrodermal tissues has been suggested to involve components of the coral immune innate repertoire, such as lectins that may play a role in the recognition and maintenance of *Symbiodinium* spp. (Wood-Charlson et al. 2006). Because coral lectins also exhibit the ability to bind a variety of bacteria (Kvennefors et al. 2008), we can speculate that the cellular process leading to the entry of bacterial endosymbionts within *P. clavata* tissues may be closely related to the uptake of *Symbiodinium* and rely on the octocoral innate immune system (Burge et al. 2013). Additional host-driven factors, such as the production of selective antimicrobial compounds, might contribute to recruit stable and specific symbionts from the environment (Franzenburg et al. 2013). Bacterial endosymbionts might have also evolved various strategies to colonize their particular host partner (Bright and Bulgheresi 2010). Consistent with the possibility of an intimate coadaptation between coral host and symbiont (La Rivière et al. 2015), the recent genomic analysis of a coral-associated *Endozoicomonas* bacterium suggested its ability to interfere with host cellular signaling to facilitate the entry into tissue layers (Ding et al. 2016).

In conclusion, this study documents the spatial distribution of tissue-associated bacteria in the gastrodermis of a gorgonian coral and may provide a valuable guide to further address the nature of interactions between endobacteria and their host. The consistent retrieval of similar bacterial associations within the gastrodermal tissues of various coral species suggests that endobacteria are an important component of the holobiont and may supply their host with additional nutritional capabilities. Future exploration of the coral–endobacterial relationships would benefit from a combination of in situ visualization approaches, such as CARD–FISH and isotopic tracing (Pett-Ridge and Weber 2012), to investigate host–microbe nutrient exchanges and their potential contribution to holobiont physiology. Such information may be essential to understand the factors mediating the establishment of endosymbiotic bacteria in coral tissues and how they influence the coral fitness in response to environmental changes.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the collection, care and use of animals were followed.

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