Intergenerational transmission of symbiotic bacteria in oviparous and viviparous demosponges, with emphasis on intracytoplasmically-compartmented bacterial types

Manuel Maldonado

Department of Aquatic Ecology, Centro de Estudios Avanzados de Blanes (CSIC), Acceso Cala St Francesc 14, Blanes 17300, Girona, Spain. E-mail: maldonado@ceab.csic.es

Recent molecular detection of vast microbial communities exclusively associated with sponges has made evident the need for a better understanding of the mechanisms by which these symbiotic microbes are handled and transferred from one sponge generation to another. This transmission electron microscopy (TEM) study investigated the occurrence of symbiotic bacteria in free-swimming larvae of two viviparous species (*Haliclona caerulea* and *Corticium candelabrum*) and spawned gametes of two oviparous species (*Chondrilla nucula* and *Petrosia ficiformis*). Complex microbial communities were found in these sponges, which in two cases included bacteria characterized by an intra-cytoplasmic membrane (ICM). When ICM-bearing and ICM-lacking bacteria co-existed, they were transferred following identical pathways. Nevertheless, the mechanism for microbial transference varied substantially between species. In *C. nucula*, a combination of intercellular symbiotic ICM-bearing and ICM-lacking bacteria, along with cyanobacteria and yeasts, were collected from the mesohyl by amoeboid nurse cells, then transported and transferred to the oocytes. In the case of *Corticium candelabrum*, intercellular bacteria did not enter the gametes, but spread into the division furrows of early embryos and proliferated in the central cavity of the free-swimming larva. Surprisingly, symbiotic bacteria were not vertically transmitted by *P. ficiformis* gametes or embryos, but apparently acquired from the environment by the juveniles of each new generation. This study failed to unravel the mechanism by which the intercellular endosymbiotic bacterium found in the central mesohyl of the *H. caerulea* larva got there. Nevertheless, the ultrastructure of this bacterial rod, which was characterized by a star-shaped cross section with nine radial protrusions, an ICM-bound riboplasm, and a putative membrane-bound acidocalcisome, suggested that it may represent a novel organization grade within the prokaryotes. It combines traits occurring in members of Poribacteria, Planctomycetes and Verrucomicrobia, emerging as one of the most complex prokaryotic architectures known to date.

INTRODUCTION

Sponges (Phylum Porifera) are known to contain a large variety of both micro-invertebrates and microbes in their bodies. Bacteria and archaea, which may occur both intracellularly and intercellularly, can reach densities as high as 109 cells g^{-1} of sponge tissue and occupy more sponge volume than do the sponge cells (e.g. Vacelet & Donadey, 1977; Wilkinson, 1978a; Santavy et al., 1990; Brantley et al., 1995; Hentschel et al., 2003). Pioneering studies suggested that the microbial communities within the sponges are different from those of the ambient seawater (e.g. Vacelet, 1975; Wilkinson, 1978b). Because a large fraction of the microbial community sampled from both sponge tissues and ambient seawater has traditionally failed—and still fails—to grow in laboratory cultures, reliable confirmation of host specificity for these microbes has remained elusive until recently. The advent of molecular techniques during the last decade has deeply transformed our understanding of bacterial diversity and phylogeny, confirming early suspicions of high specificity for many (but not all) sponge-associated microbes

(e.g. Santavy & Colwell, 1990; Hentschel et al., 2002, 2003; Imhoff & Stöhr, 2003; Enticknap et al., 2006; Hill et al., 2006). At the same time, a variety of studies have shown that sponge-associated bacteria and archaea are involved in production of bioactive compounds (Hentschel et al., 2001; Laroche et al., 2007) and transference of nutrients (Wilkinson & Garrone, 1980; Vacelet et al., 1995) and antioxidants (Regoli et al., 2004) to the sponges. These findings suggest that, in many cases, sponge-associated microbes are not generalists or opportunists, but engaged in real symbiotic or mutualistic relationships with the sponge hosts.

It was recently noticed (but see Vacelet, 1975) that many of these sponge-associated bacteria share a remarkable common trait, i.e. an intracytoplasmic membrane (ICM) defining a compartment that contains the DNA along with some cytoplasmic material and ribosomes (Fuerst et al., 1999). Such an atypical internal compartmentation in the domain Bacteria was initially thought to uniquely characterize the Planctomycetes division (Lindsay et al., 2001). At that time, it was puzzling that many of the newly-discovered, sponge-associated bacteria lacked polar differentiation

(i.e. absence of a polar cap), a stalk, and some additional morphological and ultrastructural features characterizing classical members of the Planctomycetes. Subsequent *in situ* hybridization with planctomycete-specific probes and 16S rRNA analyses soon demonstrated that while some of the sponge-associated bacteria characterized by an ICM were planctomycetes (Friederich et al., 2001; Webster et al., 2001; Pimentel-Elardo et al., 2003), others were not. Only then was it realized that a membrane-bound nucleoid occurs in sponge-associated microbes other than planctomycetes. According to subsequent 16S rRNA gene analyses and the available library, these 'other' sponge-associated bacteria with ICM, tentatively named Poribacteria, appear to make a relatively homogeneous evolutionary linage, branching deeply in the domain Bacteria (Fieseler et al., 2004). To date, the phylogenetic relationships between Planctomycetes and Poribacteria, as well as those to other phyla or divisions can be defined as controversial (e.g. see Fuerst, 2005). Likewise, its morphological distinction remains confused. Both Poribacteria and Planctomycetes appear to be morphologically characterized by having the DNA and part of the ribosomal cytoplasm (riboplasm) surrounded by at least an ICM and by showing a peripheral cytoplasmic region lacking ribosomes (paryphoplasm), limited between the outer cell membrane and the ICM (Fieseler et al., 2004; Fuerst, 2005). Nevertheless, some Planctomycetes posses an interior pair of membranes—i.e. the nuclear envelope—surrounding the genetic material to form a nuclear body isolated from the remaining riboplasm (Lindsay et al., 2001; Fuerst, 2005). Alternatively, some of the Poribacteria appear to have the DNA out of the region bounded by the ICM, which rather contains RNA (Fieseler et al., 2004).

At this stage, it is thought that most members of the Poribacteria may be specifically affiliated with the tissue of marine sponges, because samples taken from the seawater, sediment and invertebrates adjacent to the sponges gave negative results (Fieseler et al., 2004). Likewise, although the Planctomycetes are known from a variety of terrestrial and aquatic habitats, many of them have exclusively been described from marine sponges (Friederich et al., 2001; Webster et al., 2001; Pimentel-Elardo et al., 2003). Such distribution patterns suggest that these microbes are engaged in highly specialized symbiosis with their host sponges. If so, a special mechanism for efficient maintenance of the populations of these symbiotic bacteria across sponge generations should occur. Nevertheless, little is known about this process, including potential differences in the transmission mechanism between ICM-bearing and ICM-lacking bacteria. The current electron microscopy study investigated the occurrence of ICM-bearing and ICM-lacking bacteria in gametes, embryos and larvae of four different demosponges in an attempt to gain a better understanding of the processes involved in the intergenerational transfer of these microbes.

MATERIALS AND METHODS
Studied material

Two haplosclerid demosponges, the subtropical *Haliclona* (*Gellius*) *caerulea* (Hechtel, 1965) and the Mediterranean *Petrosia ficiformis* (Poiret, 1789) were investigated, along with Caribbean material of the chondrillid *Chondrilla nucula* Schmidt, 1862 and Mediterranean material of the homosclerophorid *Corticium candelabrum* (Schmidt, 1862). The two latter species are traditionally regarded to be cosmopolitan, but their world-wide distribution could well be an artefact resulting from the occurrence of several cryptic species (see Solé-Cava et al., 1992; Klautau et al., 1999).

More specifically, transmission electron microscopy (TEM) was conducted on free-swimming larvae of the two brooding species (i.e. *Haliclona caerulea* and *Corticium candelabrum*) and spawned gametes (oocytes and sperm) of the two oviparous species (i.e. *Chondrilla nucula* and *Petrosia ficiformis*). Tissue of adult individuals was also examined when required, except for *H. caerulea*, because logistic reasons made it impossible to obtain usable samples. A minimum of three samples (eggs, larvae or adult tissue) from different individuals were investigated to prevent misleading conclusions derived from rare or non-consistent observations. Terminology regarding sponge embryos and larvae followed Maldonado & Bergquist (2002) and Maldonado (2004). Terminology regarding general sponge cytology and morphology followed Boury-Esnault & Rützler (1997).

TEM techniques

This TEM study is based on sponge material that was chemically-fixed and embedded following standard TEM protocols for eukaryotic animal cells, rather than processed by cryosubstitution. Chemical fixation resulted in good preservation of the subcellular organization of the bacteria in three of the sponges, but gave only modest results in the case of the endosymbiotic bacterium of *H. caerulea*. Nevertheless, several reasons advised description of the material of *H. caerulea* originally collected: (1) the performance of the chemical fixation in this bacterium, without being ideal, was still good enough to support gross description of its basic subcellular structure; (2) to date the unique morphology of this peculiar bacterium has not been reported in the literature—even though a major research effort has been devoted to the study of sponge-associated microbes during the last decade—and it may represent a novel organization grade; and (3) unfortunately, although a 10-y period has elapsed since the original collection of *H. caerulea* larvae (Maldonado & Young, 1996), there has been no real chance for the author to repeat field sampling of this subtropical sponge to achieve cryosubstitution.

Fixation for conventional TEM took place about 1–2 h after sample collection. Samples were fixed in 2.5% glutaraldehyde in 0.2M Millonig's phosphate buffer (pH 7.4) and 0.14M NaCl for 1 h. They were then rinsed with phosphate buffer for 40 min, post-fixed in 2% OsO_4 in 0.2 M Millonig's phosphate buffer for 1 h. Larvae of *Haliclona caerulea* were dehydrated in a graded series of ethanol, infused with propylene oxide, and then embedded in EPON 812 resin at Harbor Branch Oceanographic Institution in 1995. In contrast, material belonging to *Corticium candelabrum*, *Chondrilla nucula* and *Petrosia ficiformis* was dehydrated in a graded acetone series, and embedded in Spurr's resin. In all cases, ultrathin sections obtained with an Ultracut Reichert-Jung ultramicrotome were mounted on gold grids and stained with 2% uranyl acetate for 30 min, then with
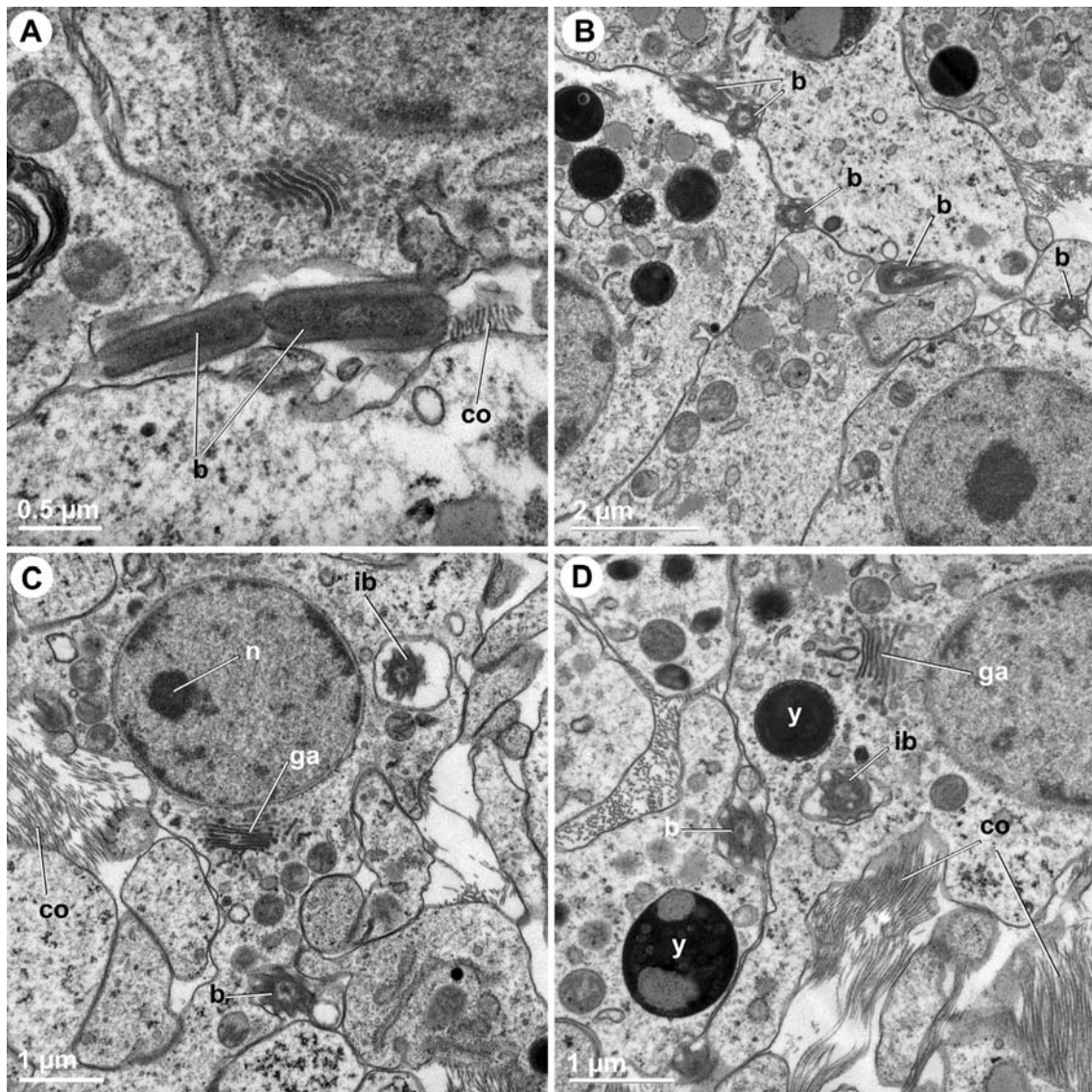
Figure 1. Intercellular and intracellular location of bacteria in the free-swimming larva of *Haliclona caerulea*. (A) Two longitudinally-sectioned rods (b) located at the intercellular spaces left between larval archeocytes, which also contained abundant collagen microfibrils (co); (B) five cross-sectioned bacteria (b) located at the narrow intercellular spaces between the archeocytes; (C&D) larval archeocytes, which were nucleolate (n) cells with a prominent Golgi apparatus (ga) and scattered yolk inclusions (y), showing intracellular bacteria (ib) in cytoplasmic, membrane-bound vacuoles. Note also occurrence of intercellular bacteria (b) and collagen microfibrils (co).

lead citrate for 10 min. Some observations on *H. caerulea* were conducted with a Philips EM-301 electron microscope, but most of the material was examined using a JEOL 1010 transmission electron microscope operating at 80 kV and provided with a Gatan module for acquisition of digital images.

RESULTS

Bacteria in Haliclona caerulea

The free-swimming larva of *Haliclona* (*Gellius*) *caerulea* was a solid, tufted parenchymella characterized by at least 11 cell types (not shown). Six cell types built the external, monolayered larval epithelium, while the internal region of the larvae contained five types of amoeboid cells (collagen-secreting collencytes, spicule-secreting sclerocytes, totipotent archaeocytes and two cell types heavily charged with diverse inclusions). These internal, non-epithelial cells allowed just narrow intercellular spaces, which were filled with collagen microfibrils and bacteria of a single morphotype.

Bacteria occurred mostly intercellularly, confined in restricted spaces left between the plasmalemma of adjacent amoeboid cells (Figure 1A,B). Never were they found between cells of the larval epithelium. Bacteria occurred isolated or in lax groups of two to five, forming neither rosettes nor other agglomerates. At the inner region of the larval body, where bacteria were more abundant, average densities were as modest as 2 ± 1.9 bacteria per 100 μm^2 of tissue (N=15). Histological sections often caught a sponge cell type, the archaeocyte, apparently phagocytosing bacteria from the intercellular medium (Figure 1C), also bearing bacteria intracellularly (Figure 1C,D). In this latter case, bacteria occurred at low density (one to three cells) within one or very few electron-clear, membrane-bound vacuoles in the archaeocyte cytoplasm, which also contained inclusions of embryonic yolk (Figure 1D). Interestingly, intracellular
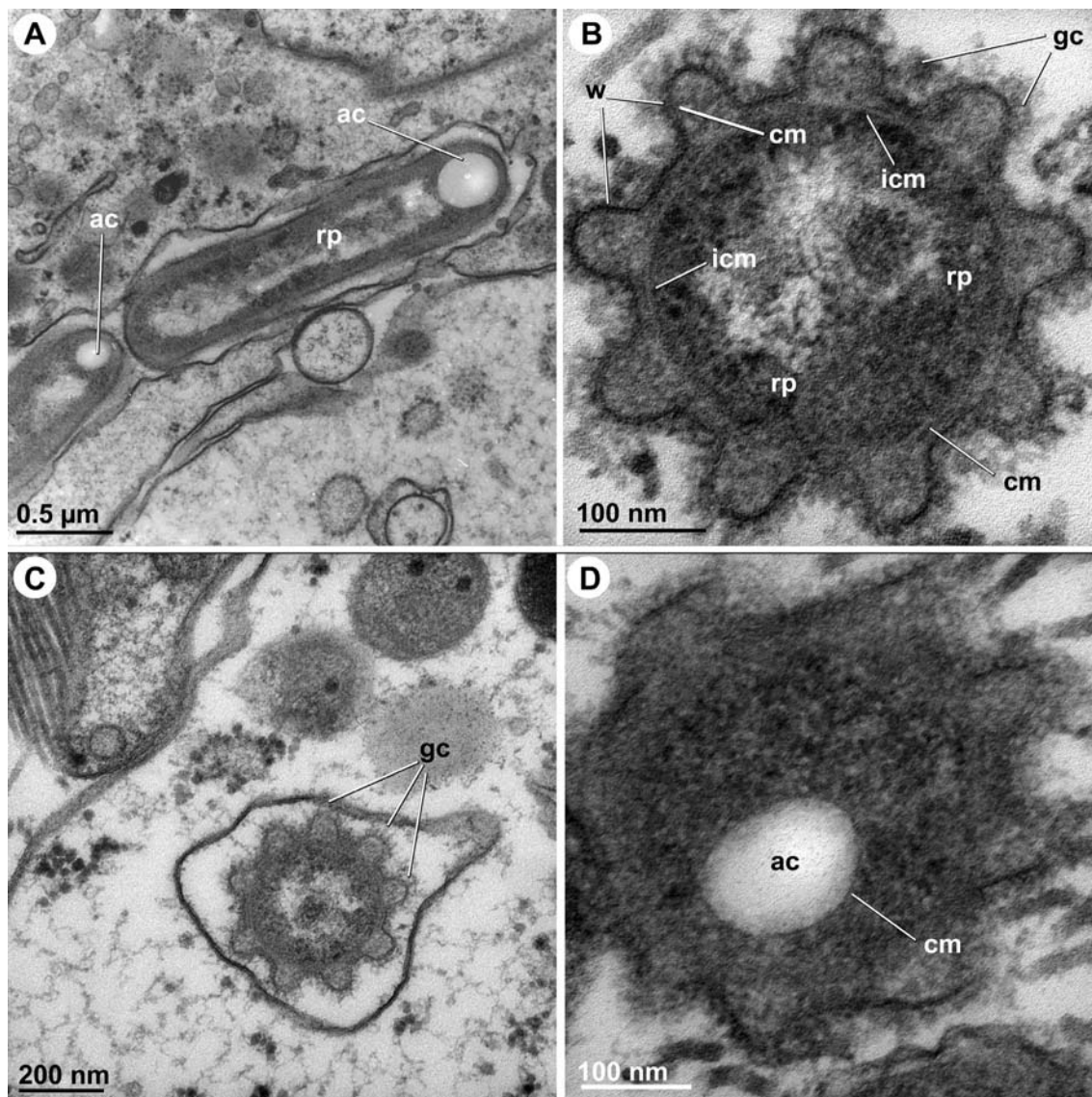
Figure 2. Morphological features of the bacterium hosted by the free-swimming larva of *Haliclona caerulea*. (A) Longitudinal section of two intercellular bacteria, showing rod morphology, an elongated riboplasm (rp), and a polar body (ac), being probably an acidocalcisome; (B) cross-sectioned, intercellular bacterium showing star-like morphology (with 9 arms), a developed gycocalyx (gc) on a thin electron-dense cell wall (w) in intimate contact with the cytoplasmic membrane (cm). An intracytoplasmic membrane (icm) envelops the large riboplasmic region (rp); (C) detail of a cross-sectioned, intracellular bacterium, showing traces of a dense glycocalyx organized in radiating projections at the tips of the wall protuberances; (D) detail of a cross-sectioned bacterium showing the putative, membrane-bound (m), acidocalcisome (ac), which is located eccentrically.

bacteria were never seen to experience digestion within the vacuoles.

In longitudinal section, these bacteria looked like regular rods (Figure 2A), measuring 1.7 to 2.4 μm in length and 0.38 to 0.52 μm in the largest diameter. Nevertheless, they were not strictly cylindrical cells, but provided with nine equidistant, longitudinal ridges, which made cross-sectioned bacteria look like a 9-arm star (Figure 2B,C). The protuberances of the bacterial cell, the height of which relative to the valleys was about 50 nm, resembled the prosthecae of both some members of Verrucomicrobia (Hedlund et al., 1997) and some star-shaped alphaproteobacteria, such as *Stella* Vasilyeva, 1985 and *Labrys* Vasilieva & Semenov, 1985 (e.g. Fritz et al., 2004). Substantial development of glycocalix (Figure 2B,C) occurred on the external side of the bacterial wall. Traces of an ICM were evident on a quite electron-

dense cytoplasmic background, limiting an elongated riboplasm, which occupied most of the cell (Figure 2A,B). Externally the cell was limited by a cytoplasmic membrane and a thin wall (Figure 2B). This bacterium showed internal polarization. At one of the ends, a pseudo-spherical, membrane-bound, electron-translucent (possibly empty) vacuole, which measured from 90 to 200 nm in diameter, occurred (Figure 2A&C). This vacuole strongly resembled—by appearance, size and position—an acidocalcisome, i.e. a membrane-bound body containing granules of phosphorus compounds.

Because tissue samples of adults could not be studied during gametogenesis and development, it remains unknown how the endosymbiotic bacterium reached the 'mesohyl' of the free-swimming larva from the parental intercellular location.
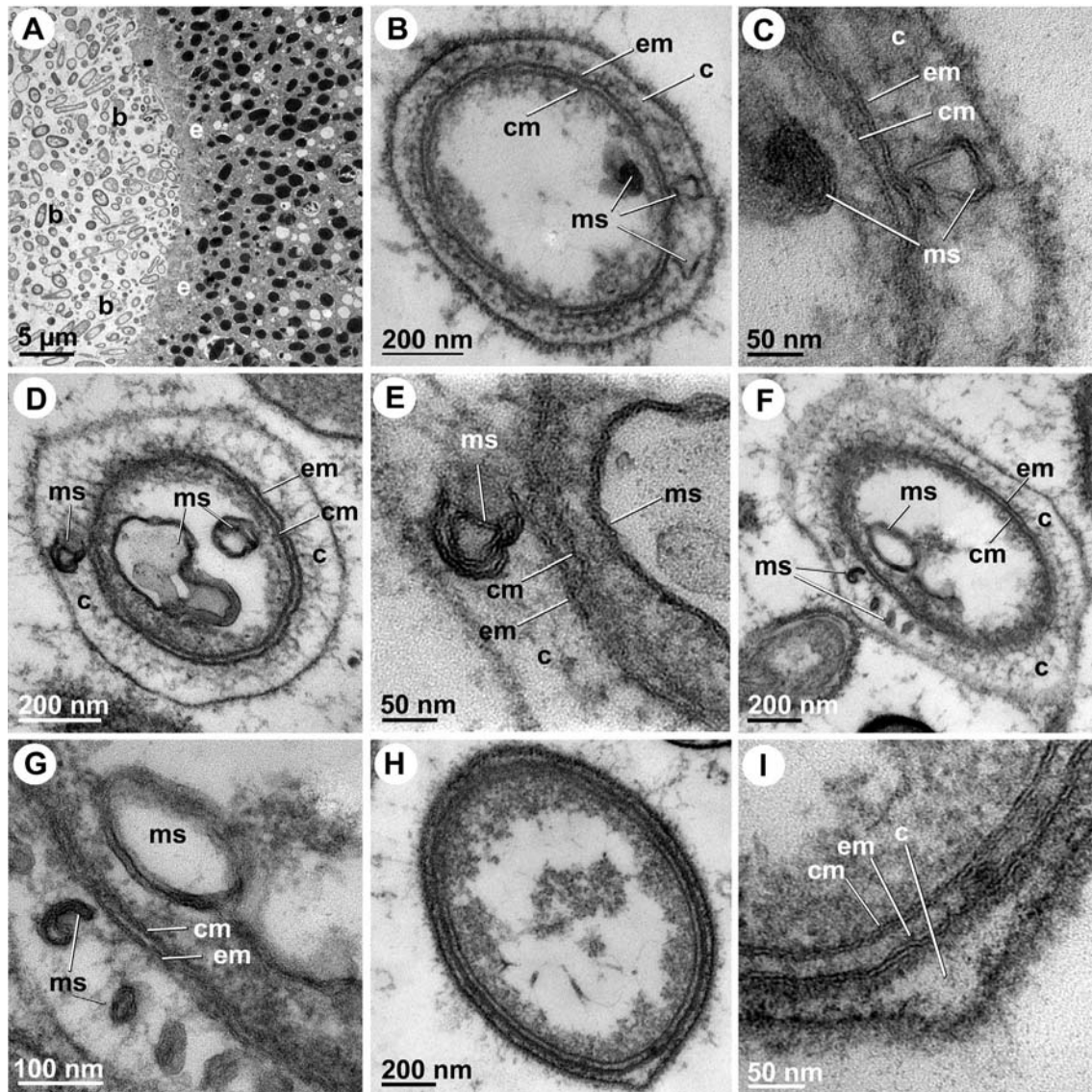
Figure 3. Bacteria in the free-swimming larva of *Corticium candelabrum*. (A) Detail of the central larval cavity filled with intercellular bacteria (b) and limited by the ciliated larval epithelium (e); (B, D, F) general views of a gram-negative morphotype characterized by a narrow wall limited between the cytoplasmic membrane (cm) and the external membrane (em), a very thick capsule (c), and mesosome-like structures (ms) putatively derived from both the external and the cytoplasmic membranes; (C, E, G) respective enlargements of the bacteria depicted in B, D and F, showing details of the membranes (cm, em), the mesosome-like structures (ms), and the capsule; (H, I) general view and detailed enlargement, respectively, of a gram-negative bacteria characterized by a narrow capsule (c) and cytoplasmic (cm) and external membranes (em) that do not form mesosomes.

Bacteria in Corticium candelabrum

The free-swimming larva of this sponge was a hollow cinctogastrula, with a monolayered epithelium consisting of three cell types (not shown). The larval epithelium delimited an internal cavity filled with collagen microfibrills and abundant bacteria (Figure 3A). At least, five different bacterial morphotypes occurred, but none showed a distinct ICM.

Some bacteria that were short, fat rods showed two membranes (Figure 3B–G). Nevertheless, the internal membrane was not an ICM, but the regular cytoplasmic membrane; the outer membrane was the external wall membrane characteristic of gram-negative bacteria. Yet this outer membrane was supplemented by an external thick capsule. The two membranes of this bacterial morphotype showed mesosome-like structures (Figure 3C,E&G) derived

from them, similar to the mesosomes described from other bacteria (e.g. Greenawalt & Whiteside, 1975). While these structures cannot be ruled out to be artefacts caused by the chemical fixation, mesosome-like deformation of the wall external membrane and the cytoplasmic membrane did not affect other gram-negative morphotypes co-occuring in these larvae (Figure 3H,I).

The transmission of the endosymbiotic bacteria to the next sponge generation involved a characteristic 'intrusion process' in which the bacteria participated more actively than did sponge cells. The intercellular endosymbiotic bacteria occurring in the mesohyl of the adult sponges progressively accumulated around the oocytes brooded by the sponges, forming a relatively thick layer around mature oocytes (Figure 4A,B). Many bacteria were phagocytosed by the oocytes, particularly during late-growth stages, but
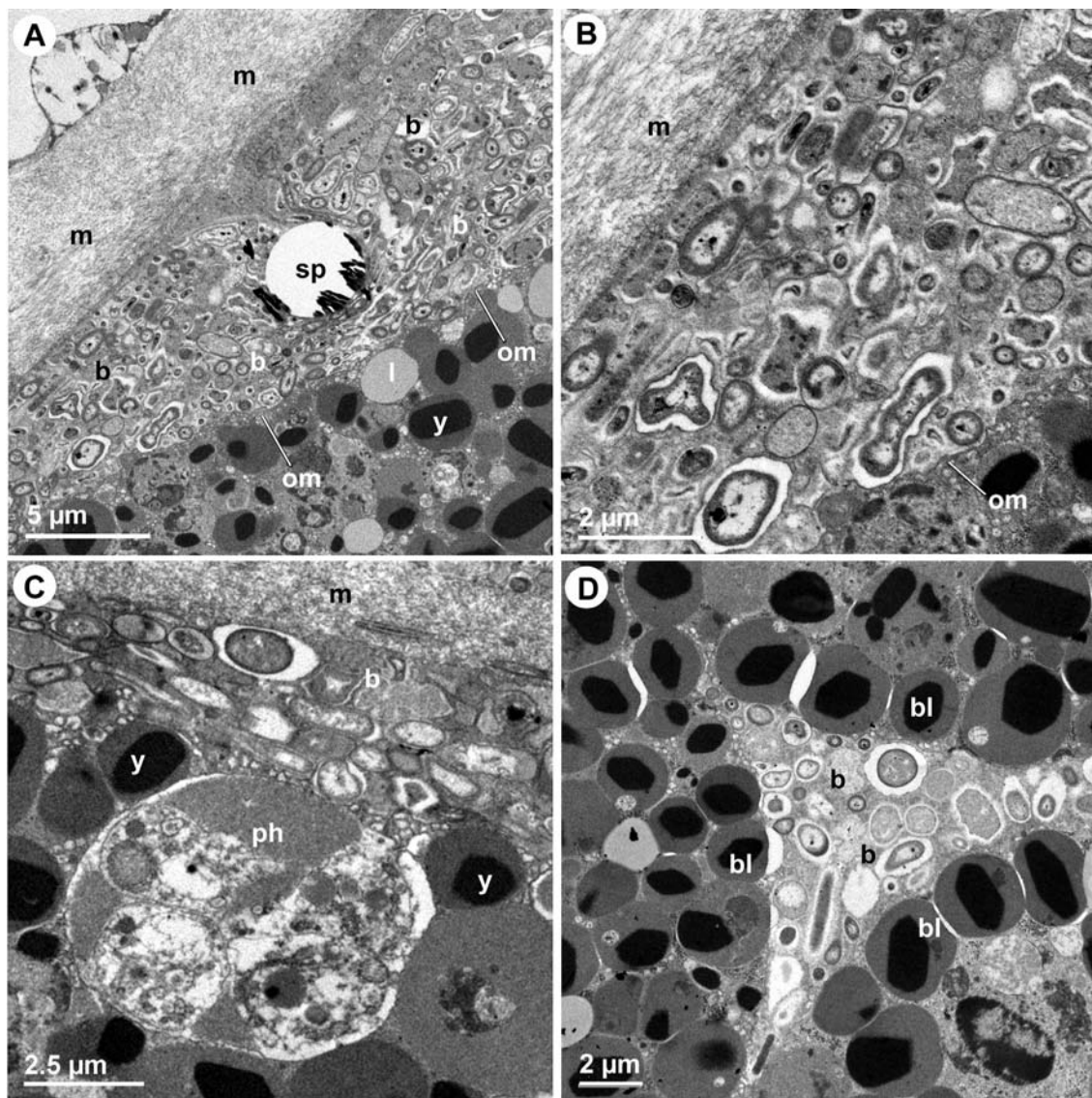
Figure 4. Bacteria around brooded eggs and embryos of *Corticium candelabrum*. (A) Bacterial layer (b) developed between the membrane of a late stage oocyte (om) and the surrounding mesohyl (m), which was very rich in collagen microfibrils. Note yolk (y) and lipid (l) bodies in the oocyte and that the bacterial layer was pierced by a spicule (sp); (B) enlarged view of A showing in greater detail the bacterial layer developed between the mesohyl (m) and the oocyte membrane (om); (C) detail of peripheral phagosome (ph) in a late-stage oocyte, the cytoplasm of which was also packed with yolk bodies (y). Note the proximity of the phagosome to the surrounding bacterial layer (b) and the adjacent mesohyl (m); (D) early blastula, in which the intercellular space left among three blastomeres (bl), had been colonized by a dense bacterial community (b).

TEM observations indicated that most were digested and used to elaborate yolk (Figure 4C). Indeed, it is likely that the bacteria ending in the central cavity of the free-swimming larva never entered the oocyte cytoplasm. Rather, they spread into the intercellular spaces of the embryo during cleavage (Figure 4D), remaining intercellular at any time during the process.

Bacteria in Chondrilla nucula

The mature oocyte of the Caribbean *Chondrilla nucula* contained an astonishing diversity of endosymbiontic microbes, including not only a variety of heterotrophic bacteria, but also cyanobacteria and yeasts (see Maldonado et al., 2005). Among the heterotrophic bacteria, two morphotypes had an ICM-bound compartment (Figure 5A).

Morphotype-1 was coccoid, measuring 0.39–0.45 μm in diameter (Figure 5A–C). It was found to divide while in the oocyte cytoplasm (Figure 5D). The ICM-bounded riboplasm was about 0.3 μm in diameter, with a central condensation of material, an intermediate region of fibrillar texture showing reticulum-like organization, and a peripheral region of material arranged in thick granules, probably containing ribosomes (Figure 5B,C). The paryphoplasm limited between the ICM and the cytoplasmic membrane was about 0.8–0.9 μm thick, with granulose appearance. Apparently, this bacterium lacked an evident wall (or it was extremely thin?), which may suggest a relationship with the primitive mycoplasms. The other ICM-compartmented bacterium (morphotype-2) was a rod, measuring up to 2 μm in length and 0.9 μm in diameter (Figure 5A,E&F). It was characterized by an ICM-bound riboplasm of ellipsoid
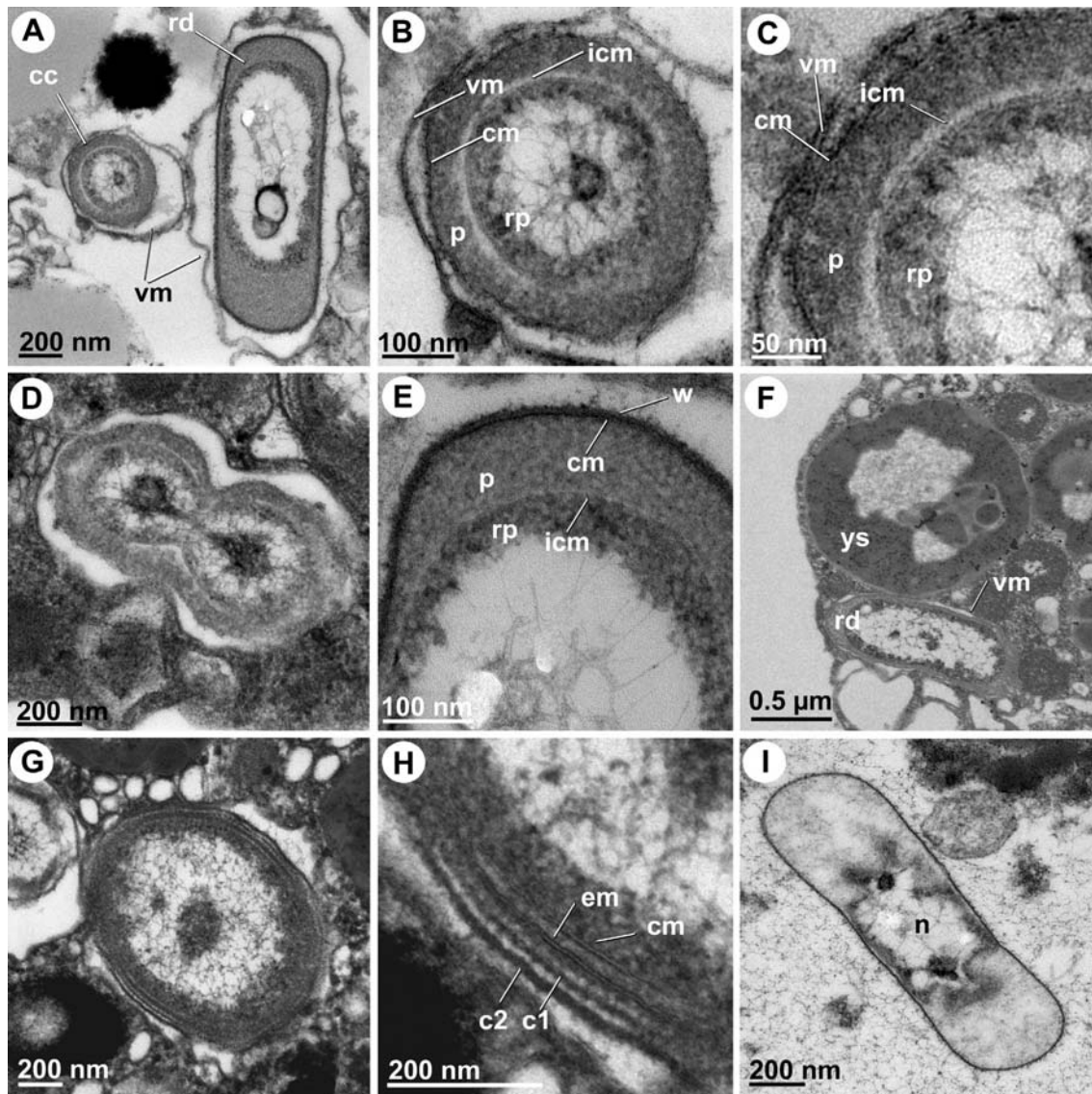
Figure 5. Bacteria in spawned oocytes of *Chondrilla nucula*. (A) ICM-bearing coccoid (cc) and rod (rd) located within membrane-bound vacuoles (vm) in the oocyte cytoplasm; (B,C) details of the coccoid morphotype showing an intracitoplasmic membrane (icm), the riboplasm (rp), a wide pariphoplasm (p), and the cytoplasmic membrane (cm). Note the vacuole membrane (vm) outside the cytoplasmic membrane and the absence of a cell wall; (D) coccoid bacterium engaged in division within the oocyte cytoplasm; (E) detail of the rod morphotype showing the intracytoplasmic membrane (icm), the cytoplasmic membrane (cm), and traces of a very thin wall (w); (F) general view of an ICM-bearing rod (rd) and symbiotic yeast (ys) in the oocyte cytoplasm. Note a vacuole membrane (vm) around the bacterium, but not around the yeast; (G,H) general view and detail of a gram-negative bacterium showing a complex envelope, in which the cytoplasmic (cm) and the external (em) membranes are externally supplemented with a bilayered capsule (c1, c2); (I) view of a gram-positive bacterium lacking ICM, but showing a butterfly-shaped nucleoid (n) similar to that described in some ICM-bearing bacteria.

section and a very thin wall (Figure 5A&E). The material of the inner region of the riboplasm was fibrillar, with reticulum-like organization, while it was condensed in thick granules at the peripheral region (Figure 5E). Both ICM-bound bacterial morphotypes were moderately abundant within the mature oocytes, consistently located in membrane-bound vacuoles (Figure 5A–D&F).

Among the non-compartmented bacteria, it is worth noting the occurrence of a short, fat rod, measuring about 1.5 µm in length and 1 µm in diameter. Its nucleoid had a central condensation and occupied most of the cell space (Figure 5G). This bacterial morphotype showed a relatively complex, electron-dense gram-negative wall, which could be misinterpreted as an ICM compartment. The periplasm,

i.e. the space located between the cytoplasmic membrane and the wall external membrane, was about 20 nm. There were also two additional envelopes external to the external wall membrane (Figure 5H). The bacterium was uncommon within the sponge tissue and occurred mostly in the cytoplasm of oocytes.

Another interesting bacterial morphotype was of a large rod lacking ICM, which occurred in both the intercellular tissue of adult sponges and oocytes. It measured up to 2.7 µm in length and 0.5 µm in diameter, showing a butterfly-shaped nucleoid filled with reticulate material which condensed peripherally (Figure 5I). This bacterium, which divided by septation (Figure 6A), was very similar to an ICM-bearing bacterium described as 'morphotype-1' from *Jaspis stellifera*
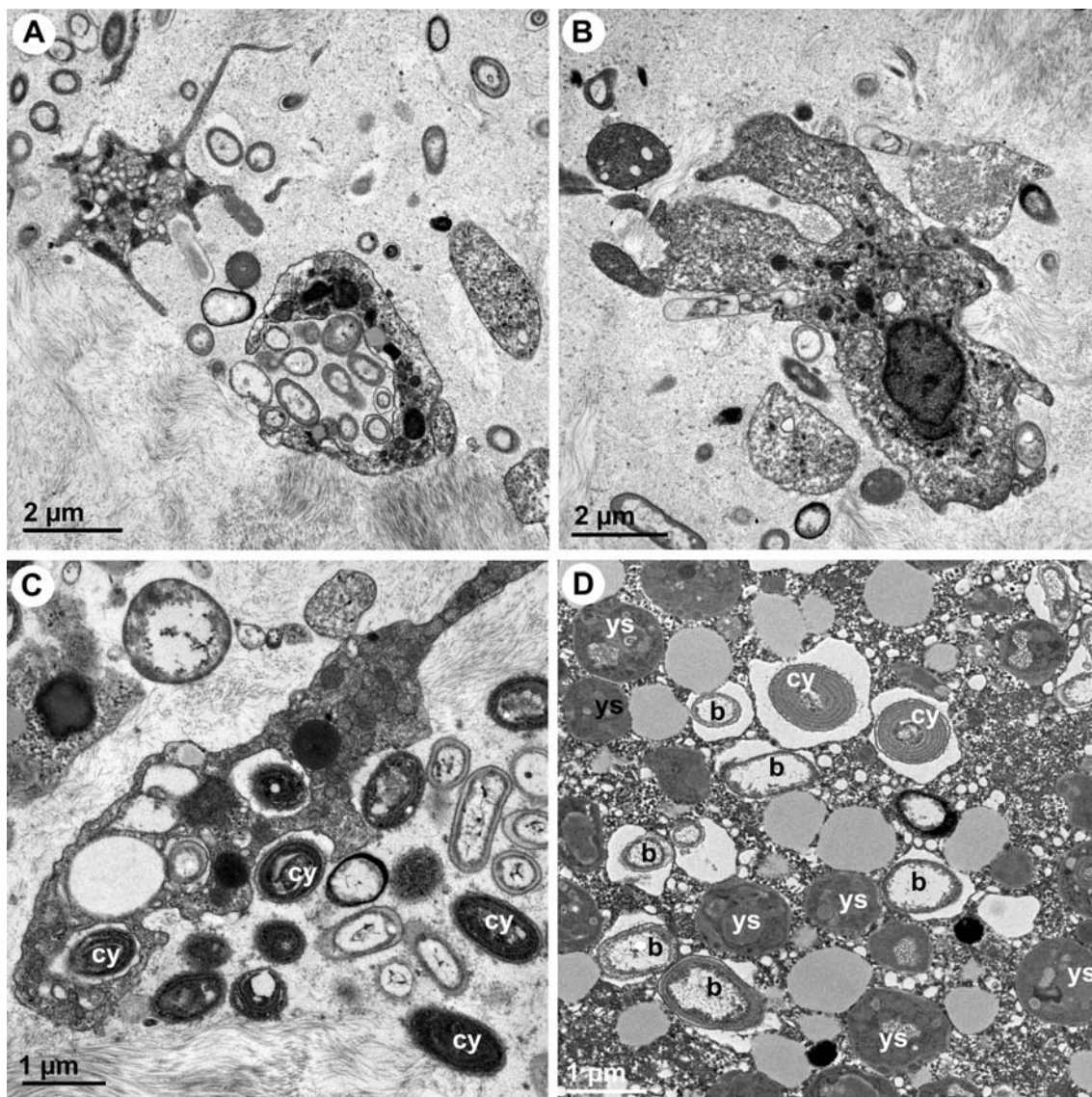
Figure 6. (A–C) Nurse cells of *Chondrilla nucula* phagocytosing bacteria and cyanobacteria (cy) from the mesohyl to store them in their cytoplasm; (D) detail of the oocyte cytoplasm showing transferred bacteria (b) and cyanobacteria (cy) in membrane-bound vacuoles; yeasts (ys) were in direct contact with the oocyte cytoplasm. For futher information on yeast identification see Maldonado et al. (2005).

by Fuerst et al. (1999) and from *Aplysina aerophoba* by Fieseler et al. (2004). The latter one, recently proposed to belong to the tentative new phylum Poribacteria (Fieseler et al., 2004), also had a butterfly nucleoid—though ICM-bound—and experienced cell division by septation.

Before entering the oocytes to become intracellular, endosymbiotic bacteria, cyanobacteria and yeasts occurred intercellularly in the mesohyl of adult sponges. There, symbiotic microbes were engulfed, but not digested, by amoeboid nurse cells (Figure 6A–C), which transported and transferred them to the cytoplasm of the growing oocytes. From the observations, it remains unclear whether oocytes engulfed entire nurse cells charged with symbionts or nurse cells fused their plasmalemma with the oocyte cytoplasmic membrane (oolemma) to release their content in the oocyte cytoplasm. Whatever the mechanism, it appears that all nurse cells enucleated before transferring their cytoplasmic charge to the oocyte, because a nucleus was never observed in late-stage nurse cells. It is also interesting that endosymbiotic

bacteria and cyanobacteria were within membrane-bound vacuoles whenever they occurred intracellularly in both nurse cells and the oocyte (Figure 6D). In contrast, endosymbiotic yeasts were in direct contact with the cytoplasm. Symbiotic microbes were never found within mature spermatozoa.

Bacteria in Petrosia ficiformis

It is long known that the adults of *Petrosia ficiformis* contain symbiotic cyanobacteria along with a variety of heterotrophic bacteria (Sará & Liaci, 1964; Vacelet & Donadey, 1977). Nevertheless, the ultrastructural examination of mature spermatozoa, oocytes and associated nurse cells in this study revealed lack of symbiotic microbes (not shown). Given that this sponge is gonochoristic and that our observations revealed that fertilization takes places externally, it must be concluded that the individuals do not experience vertical transmission of microbes. Rather, microbial populations must be incorporated from the environment at each new sponge generation.
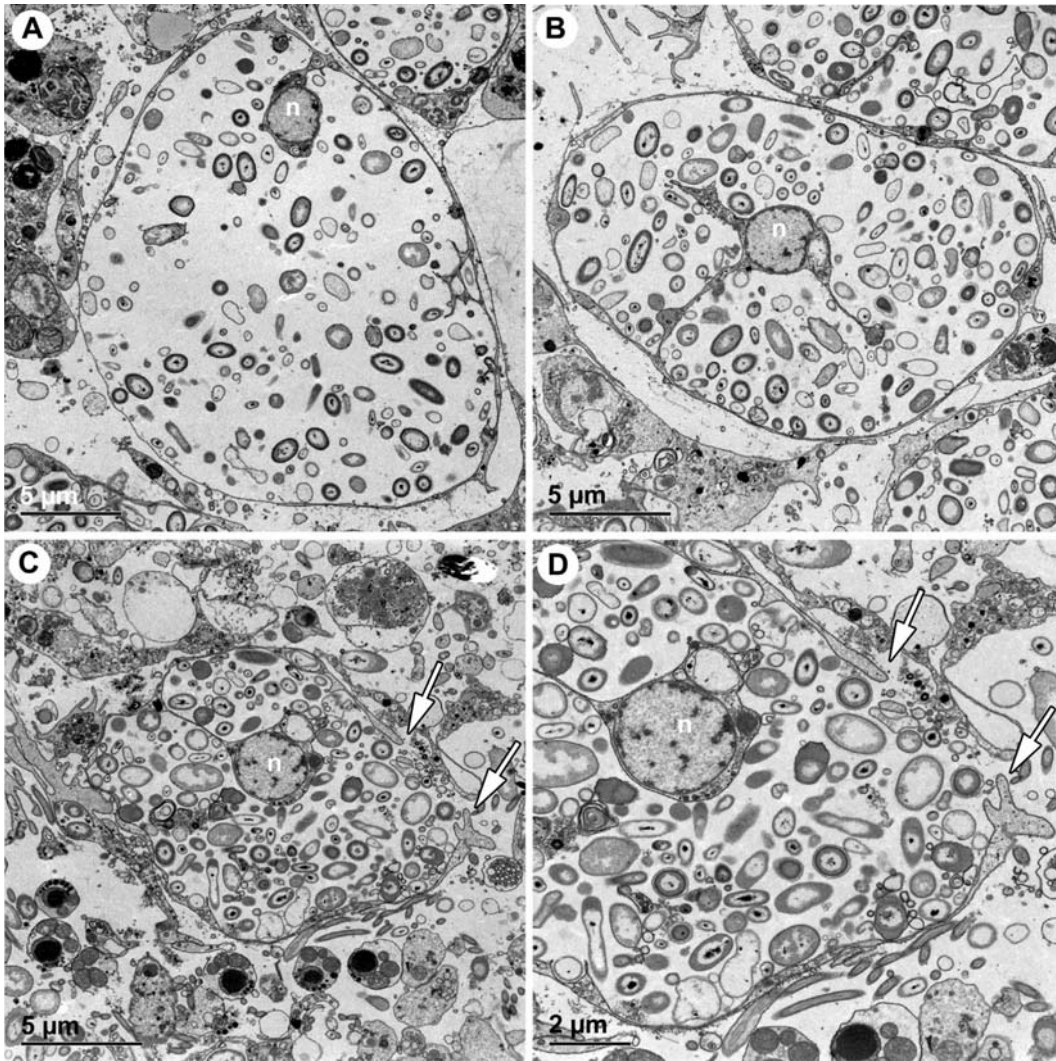
Figure 7. (A,B) Bacteriocytes of *Petrosia ficiformis* containing a large variety of bacteria within a single, large 'vacuole-like cavity'. Note the nucleus (n) protruding the cavity and the thin cytoplasmic projections making pseudo-compartments in the cavity; (C,D) general view and detail, respectively, of a bacteriocyte showing the large cavity in which bacteria are nested. The cavity, a cell pocket, is open (see arrows) to the mesohyl. Note that the nucleus (n) is protruding in the cavity, in which thin cytoplasmic projections make pseudo-compartments.
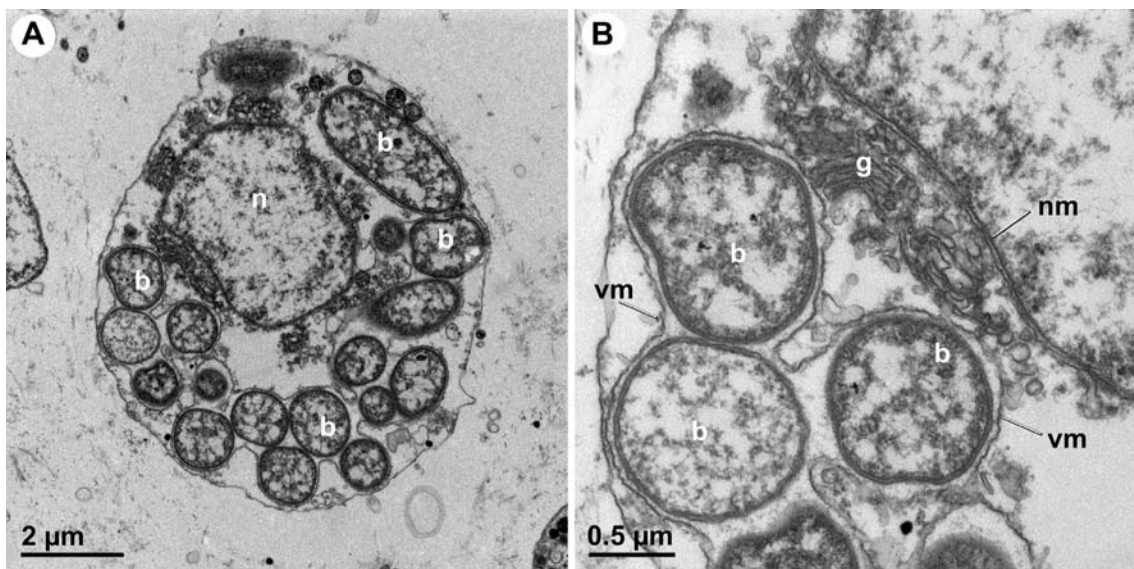

Figure 8. (A) General view of a bacteriocyte of the poecilosclerid demosponge *Crambe crambe*, showing the nucleus (n) and a set of gram-positive bacteria (b) belonging to a single morphotype; (B) detail of the bacteriocytes showing the Golgi apparatus (g) adjacent to the nuclear membrane (nm). Note that all bacteria (b) in the cell were enclosed within a single 'vacuole', the membrane of which (vm) adjusted tightly around the bacterial cells.

None of the heterotrophic bacteria occurring in the tissue of adults was found to have ICM compartmentation. These bacteria rarely occurred intercellularly in the mesohyl, but concentrated in specialized cells, known as bacteriocytes (Figure 7A–D). In the deep mesohyl, bacteriocytes hosted a mix of heterotrophic bacteria, with no preference by a given morphotype (Figure 7). In contrast to the conclusions of a previous study by Bigliardi et al. (1993), the histological sections suggested that endosymbiotic bacteria were not located in membrane-bound vacuoles of the bacteriocyte cytoplasm. Rather, it appeared that bacteria remained extracellular to the bacteriocyte, though nested in a large cavity formed by pinacocyte-like expansions of the cell, which were very long and thin (Figure 7A–D). This interpretation is supported by several observations: (1) many histological sections showed bacteriocytes having a single, large cavity (Figure 7A–C); (2) the cavity often had incomplete subdivisions originated by cell protrusions, which could lead to the wrong impression that several 'vacuoles' occurred; and (3) the cavity was often opened to the intercellular mesohyl (Figure 7D). It is also worth noting that the nucleus usually protruded into the cavity (Figure 7A–C). Such a set of features suggests that these bacteriocytes, rather than being vacuolated cells, are pinacocyte-like cells that became convex to form an extracellular cavity hosting the bacteria. The term 'pocket cells' (*sensu* Boury-Esnault & Rützler, 1997) is here proposed to refer to these special bacteriocytes.

DISCUSSION

Recent findings on sponge-associated bacteria appear to support the idea that groups of both ICM-bearing and ICM-lacking bacteria are involved in highly specialized symbiosis with sponges (e.g. Fieseler et al., 2004; Enticknap et al., 2006; Sharp et al., 2007). Therefore, it is not surprising that these microbes experience vertical transmission. Our results also show that the mechanisms used for intergenerational transference of symbiotic bacteria may differ greatly between sponge species. Nevertheless, for a given sponge species, ICM-bearing and ICM-lacking bacteria appear to follow the same transmission pathways.

It was not always clear how these bacteria entered the gametes and/or embryos. In *Haliclona caerulea*, it remains unknown how bacteria of a single morphotype got to the intercellular spaces of the larva, since the embryogenesis could not be investigated. In the case of *Chondrilla nucula*, a large variety of intercellular endosymbiotic bacteria were engulfed, along with cyanobacteria and yeasts (see Maldonado et al., 2005), by amoeboid nurse cells at different regions of the sponge mesohyl, then transported and transferred to the cytoplasm of the growing oocytes. Although we did not obtain histological sections showing cytoplasmic bridges between the nurse cells and the oocyte microvilli, such structures have been reported in other species of *Chondrilla*, such as *Chondrilla australiensis* (Usher et al., 2005). Therefore, a similar mechanism is likely to occur in the Caribbean *C. nucula*. At least 21 bacterial phylotypes have been identified in the mesohyl of the Caribbean *C. nucula* on the basis of the 16S rDNA gene (Hill et al., 2006). Although it was not clear how many of these bacteria were vertically transmitted, it was suspected that most of them

were, since they did not appear to occur free in ambient water of the sponge habitat. In some species of *Chondrilla* (e.g. *C. australiensis*) bacteria and cyanobacteria have been reported to enter not only the eggs, but also about 1.6% of the spermatozoa (Usher et al., 2005). The mechanism by which these symbionts ended up in the sperm cytoplasm remains enigmatic since spermatogenesis, unlike oogenesis, is a process addressed to minimize the cell cytoplasm (e.g. Riesgo et al., 2007). Examination of about 25 *Chondrilla nucula* spermatozoa in the current study revealed no symbiont in their cytoplasm.

A different mechanism of bacterial transmission operated in the case of *Corticium candelabrum*. Symbiotic bacteria, which accumulated around developing embryos, were able to spread into the intercellular spaces left between the blastomeres after each division. This mechanism appears to take place in homosclerophorid demosponges (Ereskovsky & Boury-Esnault, 2002; Boury-Esnault et al., 2003; Riesgo et al., 2007), also in some dictyoceratids (Kaye, 1990) and the halisarcid demosponge *Halisarca dujardini* (Ereskovsky et al., 2005).

Another mechanism of vertical transmission of microbes was described in the chondrosid demosponge *Chondrosia reniformis* (Lévi & Lévi, 1976). In this species, the symbiotic microbes were engulfed by nurse cells, which stored them in their cytoplasm. Unlike in *Chondrilla*, the nurse cells did not transfer the symbionts to the oocytes. Rather, they formed, in combination with other cell types, a cellular follicle that surrounded each mature oocyte in the maternal tissue. Oocytes surrounded by follicle cells charged with symbionts were then released to the ambient water to experience external fertilization. Upon fertilization, segmentation and embryogenesis took place successively to produce a solid blastula, then a hollow embryo. During those developmental stages the bacteria-bearing follicle cells disassembled the follicle and entered the internal cavity of the embryo through the division furrows. At this stage the externally-developing embryo became a free-swimming larva and the bacteria contained in the nurse cells were released to the larval cavity. These bacteria were subsequently found at intercellular locations in the juvenile sponges (Lévi & Lévi, 1976). A similar mechanism of vertical transmission appears to take place in demosponges of the genus *Cliona* (Warburton, 1961; Lévi & Lévi, 1976).

It is also interesting that some sponges use a multiple pathway for intergenerational transfer of bacteria. In addition to the variety of transference mechanisms mentioned so far, the oocytes of most sponges, both oviparous and viviparous species, are able to phagocyte bacteria by themselves (e.g. Gallisian & Vacelet, 1976; Gaino, 1980; Sciscioli et al., 1989, 1991; Gaino & Sará, 1994; Ereskovsky et al., 2005; Riesgo et al., 2007). Nevertheless, the role of the phagocytosed bacteria in intergenerational transmission remains unclear in many cases, since at least an unquantifiable amount of the engulfed bacteria are seen to be digested to elaborate yolk. A more sophisticated multipathway for symbiont transmission has been reported by Kaye (1990) in some dictyoceratids, such as *Hippiospongia lachne* and several *Spongia* species. In these sponges, which brood their eggs, nurse cells charged with endosymbiotic microbes engulfed from the mesohyl establish

cytoplasmic bridges with the zygote membrane, transferring symbionts and nourishing inclusions. Additionally, when these zygotes became embryos, they developed a cell follicle though also kept connected to the surrounding tissue by a system of radiating mesohyl bridges (Kaye, 1990). These bridges, which have been documented for a variety of sponge embryos (e.g. Bergquist et al., 1979; De Vos et al., 1991; Maldonado & Bergquist, 2002), are thought to facilitate the anchoring and/or feeding of the embryos during development (e.g. Kaye, 1990). Nevertheless, it appears that these mesohyl bridges also allow symbiotic bacteria to migrate from the adult mesohyl to the intercellular spaces of the embryos (Kaye, 1990).

It was first thought that vertical transmission of cyanobacteria occurred in *P. ficiformis*, since the first approach to the sexual reproduction in this sponge reported occurrence of cyanobacteria in developing oocytes using light microscopy (Scalera-Liaci et al., 1973). A subsequent TEM study on presumably mature oocytes—which were actually mid-size oocytes—reported no bacteria in the ooplasma (Lepore et al., 1995). The current study of spawned eggs and spermatozoa further corroborates the absence of both cyanobacteria and heterotrophic bacteria in mature gametes and accompanying nurse cells. Therefore, symbiotic microbes must be acquired at each generation from the environment, being subsequently handled by special cells, the pocket bacteriocytes. Instances in which microbes engaged in apparently highly evolved interactions with their hosts are incorporated from the environment by each new host generation are common among cnidarians, flatworms, bivalves, and luminescence symbiosis of cephalopods and fish. In the case of *P. ficiformis*, the cell similarity between bacteriocytes and pinacocytes may indicate that the former are pinacocyte-derived cells. Therefore, the hypothesis that bacteria may be gathered from the ambient water by exopinacocytes and/or endopinacocytes that then become convex and leave the pinacoderms should be kept in mind for further testing. While a mix of bacteria is hosted by the bacteriocytes of *P. ficiformis*, other demosponges use bacteriocytes to store a single bacterial type out of the various that may occur in their mesohyl, such as in the case of the sponge *Crambe crambe* (Schmidt, 1862) (Figure 8A,B). The physiological reason for limiting the occurrence of one or several bacterial morphotypes to the bacteriocyte pocket remains unclear, though several theoretical reasons can be invoked. (1) The enclosing of bacteria by bacteriocytes may facilitate the control of microbial populations, preventing undesirable proliferations. Such a control may particularly be needed when 'symbiotic' bacteria are not transferred vertically, but acquired from the ambient water at each new sponge generation. (2) The sponges could exploit the metabolic abilities of some bacteria to accelerate digestion of refractory structures that need to be remodelled or eliminated, such as spongin, silica pieces, foreign inclusions, and parasitic invertebrates. Likewise, nitrogen-fixing, ammonia-oxidizing, and sulphur-oxidizing, symbiotic bacteria can be used to recycle sponge excretions and metabolic sub-products generated at specific locations in the mesohyl. To this aim, cell-enclosed bacteria can be easily transported and specifically released to the required locations. (3) Alternatively, it cannot be ruled out that some symbiotic bacteria need to be enclosed by bacteriocytes, because these cell pockets provide the only suitable environment for them to survive. If this is the case, bacteriocytes can be expected to release specific nutrients to the pockets and, perhaps, selectively uptake some of the metabolic compounds produced by their hosted bacteria.

Despite chemical fixation, several of the bacterial morphotypes investigated in this study showed traces of a bi-layered ICM limiting a putative riboplasm, a feature suggesting taxonomic affiliation to either Poribacteria or Planctomycetes. To date the relationships between the two divisions of bacteria with membrane-bound nucleoid (i.e. Poribacteria and Planctomycetes) remain unresolved. One of the influential views is that planctomycetes are a deep-branching phylum (Brochier & Philippe, 2002), which may be moderately related to the also deep-branching Verrucomicrobia and Poribacteria (e.g. Fieseler et al., 2004; Fuerst, 2005). Interestingly, the endosymbiotic bacterium hosted by the *Haliclona caerulea* larva combines morphological features occurring in members of those three groups. Yet the combination makes it distinct (possibly a new genus, if not a member of a completely new phylum) and stresses the need for further investigation. This bacterium is characterized by not only its peculiar cell shape and ICM, but also an 'inclusion' similar to the acidocalcisome described from other bacteria. Acidocalcisomes are membrane-bound vacuoles containing granules of polyphosphate and other phosphorus compounds usually associated with cations, such as Ca, K, and Mg. It is known that the electron-dense content of bacterial acidocalcisomes can be lost when using standard chemical fixation and staining methods for TEM, leaving either a very thin layer of dense material that sticks to the inner side of the membrane or a completely empty vacuole (Docampo et al., 2005). This appears to be the case in the investigated material, making useless any attempt of X-ray microanalysis for the vacuole. This putative acidocalcisome, which was external to the ICM-bound compartment, was clearly distinguished from the 'intranuclear' membrane-bound anammoxosome of some anaerobic ammonium-oxidizing chemoautotrophic planctomycetes (Lindsay et al., 2001; Fuerst, 2005). It also differed from the remaining intracellular membrane-bound structures known in prokaryotes, such as carboxysomes, enterosomes, gas vacuoles, chromatophores and various pigment granules, sulphur globules, glycogen granules, and magnetosomes. All together, the ultrastructural features of this star-shaped rod suggest that this organism may be a promising source of new information to improve our understanding of the relationships between the deep branches of the prokaryotes as well as the origin of membrane compartmentation in eukaryotic cells.

REFERENCES

Bergquist, P.R., Sinclair, M.E., Green, C.R. & Silyn-Roberts, H., 1979. Comparative morphology and behaviour of larvae of Demospongiae. In *Biologie des spongiaires* (ed. C. Lévi and N. Boury-Esnault), pp. 103–111. Paris: CNRS.

Bigliardi, E., Sciscioli, M. & Lepore, E., 1993. Interactions between prokaryotic and eukaryotic cells in sponge endocytobiosis. *Endocytobiosis and Cell Research*, **9**, 215–221.

Boury-Esnault, N., Ereskvosky, A.V., Bézac, C. & Tokina, D.B., 2003. Larval development in *Homoscleromorpha* (Porifera, Demospongiae): first evidence of a basement membrane in sponge larvae. *Invertebrate Biology*, **122**, 187–202.

Boury-Esnault, N. & Rützler, K., 1997. Thesaurus of sponge morphology. *Smithsonian Contributions to Zoology*, **596**, 1–55.

Brantley, S.E., Molinski, T.F., Preston, C.M. & DeLong, E.F., 1995. Brominated acetylenic fatty acids from *Xestospongia* sp., a marine sponge–bacterial association. *Tetrahedron*, **51**, 7667–7672.

Brochier, C. & Philippe, H., 2002. Phylogeny: a non-hyperthermophilic ancestor for bacteria. *Nature, London*, **417**, 244.

De Vos, L., Rützler, K., Boury-Esnault, N., Donadey, C. & Vacelet, J., 1991. *Atlas of sponge morphology*. Washington: Smithsonian Institution Press.

Docampo, R., De Souza, W., Miranda, K., Rohloff, P. & Moreno, S.N.J., 2005. Acidocalcisomes—conserved from bacteria to man. *Nature Reviews*, **3**, 251–261.

Enticknap, J., Kelly, M., Peraud, O. & Hill, R.T., 2006. Characterization of a culturable alphaproteobacterial symbiont common to many marine sponges and evidence for vertical transmission via sponge larvae. *Applied and Environmental Microbiology*, **72**, 3724–3732.

Ereskovsky, A.V. & Boury-Esnault, N., 2002. Cleavage pattern in *Oscarella* species (Porifera, Demospongiae, Homoscleromorpha): transition of maternal cells and symbiotic bacteria. *Journal of Natural History*, **36**, 1761–1775.

Ereskovsky, A.V., Gonobobleba, E. & Vishnyakov, A., 2005. Morphological evidence for vertical transmission of symbiotic bacteria in the viviparous sponge *Halisarca dujardini* Johnston (Porifera, Demospongiae, Halisarcida). *Marine Biology*, **146**, 869–875.

Fieseler, L., Horn, M., Wagner, M. & Hentschel, U., 2004. Discovery of the novel candidate phylum "Poribacteria" in marine sponges. *Applied and Environmental Microbiology*, **70**, 3724–3732.

Friederich, A.B., Fischer, I., Proksch, P., Hacker, J. & Hentschel, U., 2001. Temporal variation of the microbial community associated with the Mediterranean sponge *Aplysina aerophoba*. *FEMS Microbiology Ecology*, **38**, 105–113.

Fritz, I., Strömpl, C. & Abraham, W.R., 2004. Phylogenetic relationships of the genera *Stella*, *Labrys*, and *Angulomicrobium* within the "Alphaproteobacteria" and description of *Angulomicrobium amanitiforme* sp. nov. *International Journal of Systematic and Evolutionary Microbiology*, **54**, 651–657.

Fuerst, J.A., 2005. Intracellular compartmentation in Planctomycetes. *Annual Review of Microbiology*, **59**, 299–328.

Fuerst, J.A., Webb, R.I., Garson, M.J., Hardy, L. & Reiswig, H.M., 1999. Membrane-bounded nuclear bodies in a diverse range of microbial symbionts of Great Barrier Reef sponges. *Memoirs of the Queensland Museum*, **44**, 193–203.

Gaino, E., 1980. Indagine ultrastrutturale sugli ovociti maturi di *Chondrilla nucula* Schmidt (Porifera, Demospongiae). *Cahiers de Biologie Marine*, **21**, 11–22.

Gaino, E. & Sarà, M., 1994. An ultrastructural comparative study of the eggs of two species of *Tethya* (Porifera, Demospongiae). *Invertebrate Reproduction and Development*, **26**, 99–106.

Gallisian, M.F. & Vacelet, J., 1976. Ultrastructure de quelques stades de l'ovogénèse des spongiaires du genre *Verongia* (Dictyoceratida). *Annales des Sciences Naturelles. Zoologie et Biologie Animale*, **18**, 381–404.

Greenawalt, J.W. & Whiteside, T.L., 1975. Mesosomes: membranous bacterial organelles. *Bacteriological Reviews*, **39**, 405–463.

Hedlund, B.P., Gosink, J.J. & Staley, J.T., 1997. Verrucomicrobia div. nov., a new division of the bacteria containing three new species of *Prosthecobacter*. *Antonie van Leeuwenhoek*, **72**, 29–38.

Hentschel, U., Fieseler, L., Wehrl, M., Gernet, C., Steinert, M., Hacker, J. & Horn, M., 2003. Microbial diversity of marine sponges. In *Sponges (Porifera)* (ed. W.E.G. Müller), pp. 59–88. Berlin: Springer-Verlag.

Hentschel, U., Hopke, J., Horn, M., Friederich, A.B., Wagner, M., Hacker, J. & Moore, B.S., 2002. Molecular evidence for a uniform microbial community in sponges from different oceans. *Applied and Environmental Microbiology*, **68**, 4431–4440.

Hentschel, U., Schmid, M., Wagner, M., Fieseler, L., Gernet, C. & Hacker, J., 2001. Isolation and phylogenetic analysis of bacteria with antimicrobial activities from the Mediterranean sponges *Aplysina aerophoba* and *Aplysina cavernicola*. *FEMS Microbiology Ecology*, **35**, 305–312.

Hill, M.S., Hill, A.L., López, N. & Harriot, O., 2006. Sponge-specific bacterial symbionts in the Caribbean sponge *Chondrilla nucula* (Demospongiae, Chondrosida). *Marine Biology*, **148**, 1221–1230.

Imhoff, J.F. & Stöhr, R., 2003. Sponge-associated bacteria: general overview and special aspects of bacteria associated with *Halichondria panicea*. In *Sponges (Porifera)* (ed. W.E.G. Müller), pp. 35–57. Berlin: Springer-Verlag.

Kaye, H.R., 1990. Sexual reproduction in four Caribbean commercial sponges. II. Oogenesis and transfer of bacterial symbionts. *Invertebrate Reproduction and Development*, **19**, 13–14.

Klautau, M., Russo, C., Lazoski, C., Boury-Esnault, N., Thorpe, J.P. & Solé-Cava, A.M., 1999. Does cosmopolitanism in morphologically simple species result from overconservative systematics? A case study using the marine sponge *Chondrilla nucula*. *Evolution*, **53**, 1414–1422.

Laroche, M., Imperatore, C., Grozdanov, L., Costantino, V., Mangoni, A., Hentschel, U. & Fattorusso, E., 2007. Cellular localisation of secondary metabolites isolated from the Caribbean sponge *Plakortis simplex*. *Marine Biology*, **151**, 1365–1373.

Lepore, E., Sciscioli, M. & Scalera-Liaci, L., 1995. The ultrastructure of the mature oocyte and the nurse cells of the ceractinomorpha *Petrosia ficiformis*. *Cahiers de Biologie Marine*, **36**, 15–20.

Lévi, C. & Lévi, P., 1976. Embryogénèse de *Chondrosia reniformis* (Nardo), démosponge ovipare, et transmission des bactéries symbiotiques. *Annales des Sciences Naturelles (Zoologie)*, **18**, 367–380.

Lindsay, M.R., Webb, R.I., Strous, M., Jetten, M.S.M., Butler, M.K., Forde, R.J. & Fuerst, J.A., 2001. Cell compartmentalisation in planctomycetes: novel types of structural organisation for the bacterial cell. *Archives of Microbiology*, **175**, 413–429.

Maldonado, M., 2004. Choanoflagellates, choanocytes, and animal multicellularity. *Invertebrate Biology*, **123**, 231–242.

Maldonado, M. & Bergquist, P.R., 2002. Phylum Porifera. In *Atlas of marine invertebrate larvae* (ed. C.M. Young et al.), pp. 21–50. San Diego: Academic Press.

Maldonado, M., Cortadellas, N., Trillas, M.I. & Rützler, K., 2005. Endosymbiotic yeast maternally transmitted in a marine sponge. *Biological Bulletin. Marine Biological Laboratory, Woods Hole*, **209**, 94–106.

Maldonado, M. & Young, C.M., 1996. Effects of physical factors on larval behavior, settlement and recruitmet of four tropical demosponges. *Marine Ecology Progress Series*, **138**, 169–180.

Pimentel-Elardo, S., Wehrl, M., Friederich, A.B., Jensen, P.R. & Hentschel, U., 2003. Isolation of planctomycetes from *Aplysina* sponges. *Aquatic Microbial Ecology*, **33**, 239–245.

Regoli, F., Cerrano, C., Chierici, E., Chiantore, M.C. & Bavastrello, G., 2004. Seasonal variability of pro-oxidant pressure and antioxidant adaptation to symbiosis in the Mediterranean *Petrosia ficiformis*. *Marine Ecology Progress Series*, **275**, 129–137.

Riesgo, A., Maldonado, M. & Durfort, M., 2007. Dynamics of gametogenesis, embryogenesis, and larval release in a Mediterranean homosclerophorid demosponge. *Marine and Freshwater Research*, **58**, 398–417.

Santavy, D.L. & Colwell, R.R., 1990. Comparison of bacterial communities associated with the Caribbean sclerosponge *Ceratoporella nicholsoni* and ambient seawater. *Marine Ecology Progress Series*, **67**, 73–82.

Santavy, D.L., Willenz, P. & Colwell, R.R., 1990. Phenotypic study of bacteria associated with the Caribbean sclerosponge, *Ceratoporella nicholsoni*. *Applied and Environmental Microbiology*, **56**, 1750–1762.

Sarà, M. & Liaci, L.S., 1964. Symbiotic association between zooxanthellae and two marine sponges of the genus *Cliona*. *Nature, London*, **4942**, 321.

Scalera-Liaci, L., Sciscioloi, M. & Matarrese, A., 1973. Raffronto tra il comportamento sessuale di alcune *Ceractinomorpha*. *Revista di Biologia*, **66**, 135–162.

Sciscioli, M., Lepore, E., Scalera-Liaci, L. & Gherardi, M., 1989. Indagine ultrastrutturale sugli ovociti di *Erylus discophorus* (Schmidt) (Porifera, Tetractinellida). *Oebalia*, **15**, 939–941.

Sciscioli, M., Scalera-Liaci, L., Gherardi, M. & Simpson, T.L., 1991. Ultrastructural study of the mature egg of the marine sponge *Stelleta grubii* (Porifera, Demospongiae). *Molecular Reproduction and Development*, **28**, 346–350.

Sharp, K.H., Eam, B., Faulkner, J. & Haygood, M.G., 2007. Vertical transmission of diverse microbes in the tropical sponge *Corticium* sp. *Applied and Environmental Microbiology*, **73**, 622–629.

Solé-Cava, A.M., Boury-Esnault, N., Vacelet, J. & Thorpe, J.P., 1992. Biochemical genetic divergence and systematics in sponges of the genera *Corticium* and *Oscarella* (Demospongiae: Homoscleromorpha) in the Mediterranean Sea. *Marine Biology*, **113**, 299–304.

Usher, K.M., Sutton, D.C., Toze, S., Kuo, J. & Fromont, J., 2005. Inter-generational transmission of microbial symbionts in the marine sponge *Chondrilla australiensis* (Demospongiae). *Marine and Freshwater Research*, **56**, 125–131.

Vacelet, J., 1975. Étude en microscopie électronique de l'association entre bactéries et spongiaires du genre *Verongia* (Dictyoceratida). *Journal de Microscopie et de Biologie Cellulaire*, **23**, 271–288.

Vacelet, J., Boury-Esnault, N., Fiala-Medioni, A. & Fisher, C.R., 1995. A methanotrophic carnivorous sponge. *Nature, London*, **377**, 296.

Vacelet, J. & Donadey, C., 1977. Electron microscope study of the association between some sponges and bacteria. *Journal of Experimental Marine Biology and Ecology*, **30**, 301–314.

Warburton, F.E., 1961. Inclusion of parental somatic cells in sponge larvae. *Nature, London*, **191**, 1317.

Webster, N.S., Wilson, K.J., Blackall, L.L. & Hill, R.T., 2001. Phylogenetic diversity of bacteria associated with the marine sponge *Rhopaloeides odorabile*. *Applied and Environmental Microbiology*, **67**, 434–444.

Wilkinson, C.R., 1978a. Microbial associations in sponges. II. Numerical analysis of sponge and water bacterial populations. *Marine Biology*, **49**, 169–176.

Wilkinson, C.R., 1978b. Microbial associations in sponges. III. Ultrastructure of the *in situ* associations in coral reef sponges. *Marine Biology*, **49**, 177–185.

Wilkinson, C.R. & Garrone, R., 1980. Nutrition of marine sponges. Involvement of symbiotic bacteria in the uptake of dissolved carbon. In *Nutrition in lower metazoans* (ed. D.C. Smith), pp. 157–161. Oxford: Oxford University Press.