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Microbial Associations in Sponges. III. Ultrastructure of the *in situ* Associations in Coral Reef Sponges

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Abstract

Symbiotic cyanobacteria are associated with marine sponges in three ways: the majority are free-living in the mesohyl; large aggregates occur in "cyanocytes" (specialized, vacuolated archeocytes); and few are present in digestive vacuoles. The cyanobacteria in *Jaspis stellifera* and *Neofibularia irata* are morphologically similar to those described in Mediterranean sponges, whereas those in *Pericharax heteroraphis* are different. The free-living bacterial populations are morphologically similar, although the number of bacteria varies between the species. The fourth sponge *Ircinia wistarii* contains a mixed bacterial population unlike those in the other sponges. Sponge digestion of microbial associates is rare and not considered to contribute significant nutrients.

Introduction

Although large numbers of microorganisms have been observed in many marine sponges, few authors have examined these microorganisms in detail. Feldman (1933) classified the cyanobacteria (blue-green algae) he saw in Mediterranean sponges as genus *Aphanocapsa*. Sarà (1971) attempted to speciate the cyanobacteria in *Ircinia variabilis*. However, the only detailed description of sponge cyanobacteria was given by Vacelet (1971) during examination of *Verongia aerophoba*.

The nature of granular particles within sponge mesohyl was disputed until Lévi and Porte (1962) used an electron microscope to confirm that they were bacteria. Since then bacteria have been observed in the nucleus of *Verongia* spp. cells and as dense masses in the mesohyl (Vacelet, 1970, 1975). Five morphological bacterial types were described and these were stated to form three types of association within sponges (Vacelet, 1975).

The electron microscope was used to examine the microbial associates in 4 Great Barrier Reef sponges: 1 Calcarea, *Pericharax heteroraphis*; and 3 Demospongiae, *Jaspis stellifera*, *Neofibularia irata* and *Ircinia wistarii*. Large bacterial populations and varying amounts of cyanobacterial chlorophyll *a* were detected previously in these sponges (Wilkinson, 1978a).

These ultrastructural examinations were performed to compare the microbial populations between sponges and with those described elsewhere. In particular, the sponge bacteria were compared with the isolates which were found to be the predominant symbionts in the sponges (Wilkinson, 1978b). Furthermore it was hoped that an understanding of the nature of the microbial/sponge association and the possible roles of the symbionts could be obtained.

Materials and Methods

Whole sponge specimens were collected over a 2 year period using SCUBA apparatus, and small portions were fixed within 20 min of collection in either 3% glutaraldehyde in 0.5 M phosphate buffer with rinses in 1% phosphate-buffered sucrose, or 4% glutaraldehyde in 0.05 M Na cycodylate, 0.002 M Ca acetate and 0.67 M sucrose with rinses in the above without glutaraldehyde. The tissue was post-fixed in 2% osmium tetroxide and embedded in Spurr's resin.

Five bacterial strains (*Pericharax heteroraphis*, P85; *Jaspis stellifera*, J69; *Neofibularia irata*, G72; *Ircinia wistarii*, I46 and I78) selected from the major numerical cluster groups obtained from strains isolated from the sponges (Wilkinson, 1978b), were grown on membrane filters

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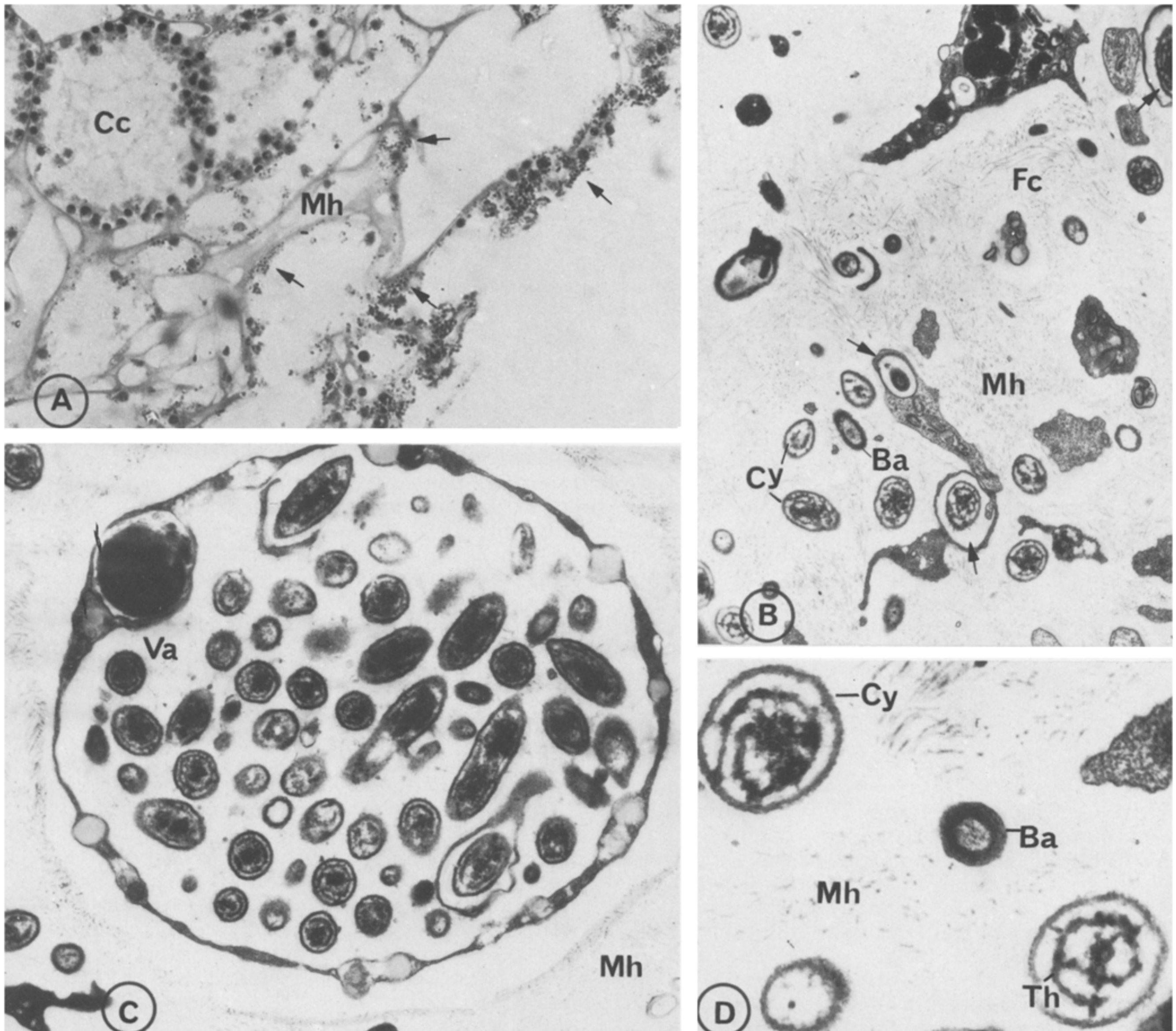
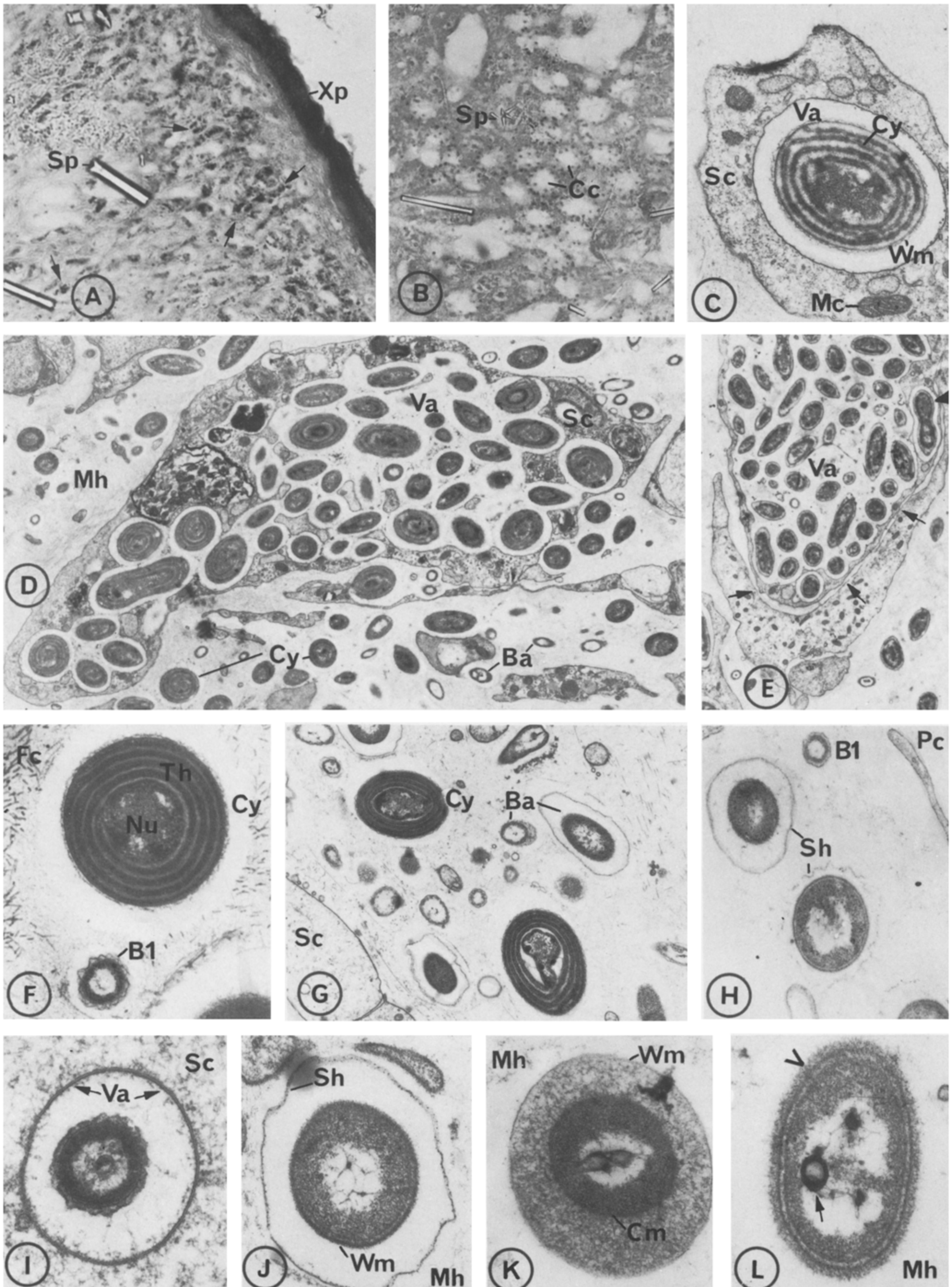


Fig. 1. *Pericharax heteroraphis.* (A) Ectosome showing aggregates of cyanobacteria (small dark spots, arrowed) adjacent to choanocyte chambers (Cc) (x 300); (B) free-living and vacuolated (arrowed) cyanobacteria (Cy) and bacteria (Ba) in ectosome collagenous mesohyl (x 8,200); (C) large cyanocyte with many cyanobacteria and few bacteria in the same vacuole (Va) (x 11,500); (D) detail of cyanobacteria with spiral thylakoid (Th) (x 28,700). Fc: fibres of collagen; Mh: mesohyl

Fig. 2. *Jaspis stellifera.* (A) Ectosome showing dense aggregates of cyanobacteria (arrowed) behind exopinacoderm (Xp) (x 300); (B) endosome with choanocyte chambers (Cc) but no cyanobacterial aggregates (x 300); (C) cyanobacterium (Cy) in sponge cell vacuole (Va) – note undulating cell wall membrane (Wm) (x 15,800); (D) cyanocyte with one large vacuole containing numerous cyanobacteria interspersed with sponge cytoplasmic pseudopodia – note free-living cyanobacteria and bacteria (Ba) in mesohyl (Mh) (x 6,550); (E) cyanocyte partially surrounded by and in close contact with another sponge amoeboid cell (small arrows) – note dividing cyanobacterium in separate vacuole (arrowhead) (x 4,600); (F) cyanobacteria and Type 1 bacterium (B1) with capsule-like clearings in mesohyl (x 28,000); (G) cyanobacteria, bacteria and sponge cells in close proximity within ectosome mesohyl (x 11,900); (H) intact and disrupted sheaths (Sh) around Type 2 bacteria near canal pinacocyte (Pc) – compare size of adjacent Type 1 cell (B1) (x 19,700); (I) Type 1 bacterium within sponge cell vacuole (arrowed) – note undulating cell wall membrane (x 56,800); (J) Type 2 bacterium with sheath well separated from bacterial cell wall (Wm) (x 37,400); (K) Type 4 bacterium with large separation between cytoplasmic (Cm) and cell wall membrane (x 40,000); (L) Type 5 bacterium with outer microcapsule (V) and internal mesosome (arrowed) (x 51,500). Fc: fibres of collagen; Mc: mitochondrion; Nu: nucleoplasm; Sc: sponge cytoplasm; Sp: spicule; Th: thylakoid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on nutrient sea water agar, and complete colonies were fixed in 2½% glutaraldehyde in 85% artificial sea water (Wilkinson, 1978b), post-fixed in 2% osmium tetroxide, and embedded in Epon resin.

Ultra-thin sections were stained with uranyl acetate:lead citrate and examined using either a Jeol JEM 100U or a Hitachi HU12 electron microscope. Cell walls were measured to include both cytoplasmic and cell wall membranes.

Sponge samples were fixed in acetic acid-Bouin's fluid for light microscopy. *Pericharax heteroraphis* was decalcified in Decal bone decalcifier and all tissue was treated by standard histological methods and sections stained with haematoxylin-eosin.

Results

Cyanobacteria

Cyanobacteria were evident as highly refractile particles in the illuminated ectosomes of *Pericharax heteroraphis* and *Jaspis stellifera* (Figs. 1A, 2A) and throughout the tissue of *Neofibularia irata*. No cyanobacteria were observed in *Ircinia wistarii*. These cyanobacteria were shown by electron microscopy to occur in 3 specific ways within the sponges: (1) free-living in the mesohyl (Figs. 1B, 2G); (2) singly or as pairs in small, closed, sponge cell vacuoles (Figs. 2C, 3A); (3) as large aggregations within large, sponge cell vacuoles (Figs. 1C, 2D). The

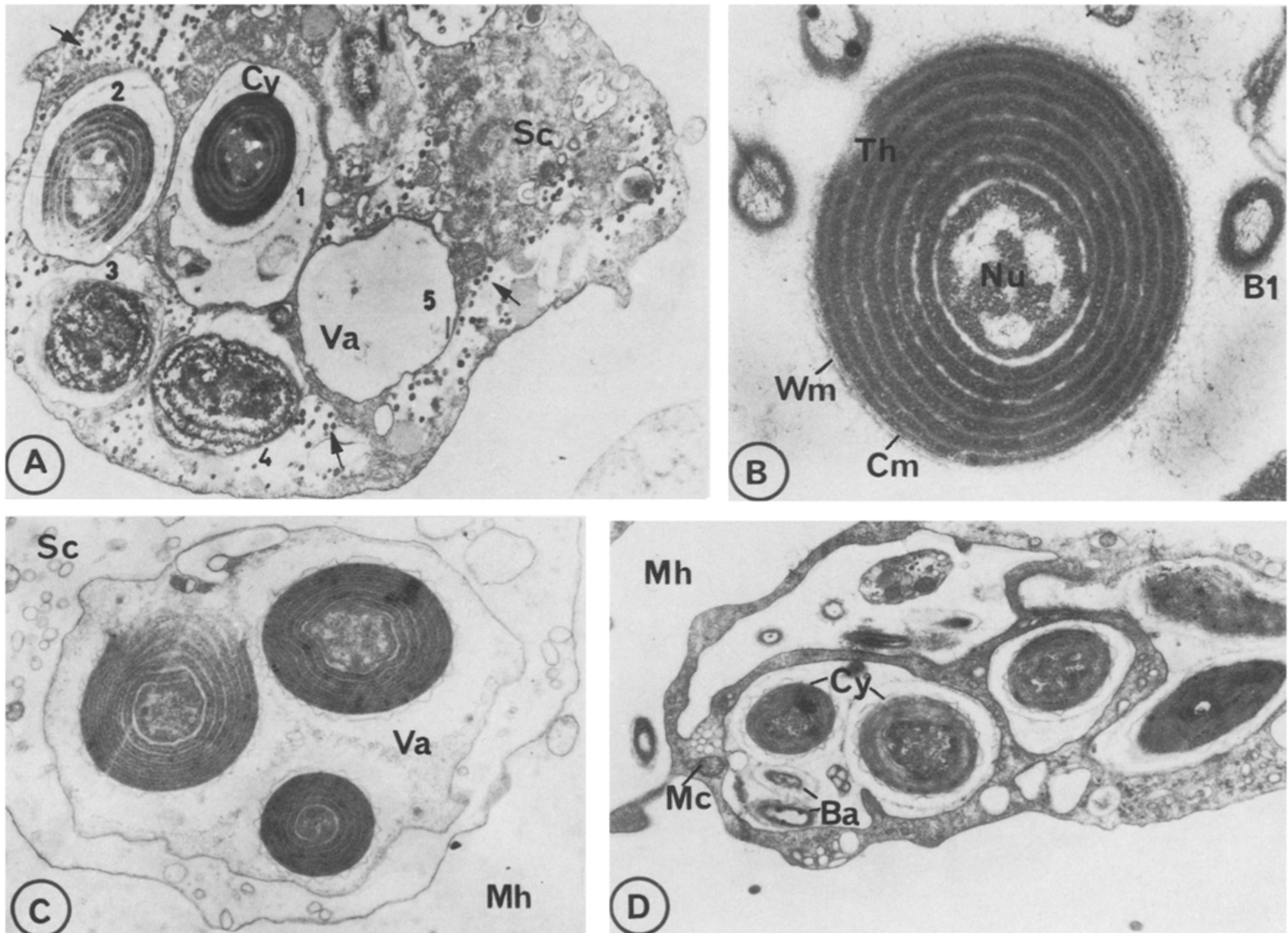

Fig. 3. *Neofibularia irata*. (A) Vacuolated sponge archeocyte containing cyanobacteria (Cy): 1, intact cell; 2, lysed cell; 3, partially digested cell with disrupted thylakoid; 4, as 3 with vacuole membrane absorbed; 5, empty vacuole (Va) — note granular material resembling glycogen particles (arrowed) (x 12,600). (B) Cyanobacterium with 7 thylakoid turns; Type 1 bacterium (B1) is adjacent (x 40,000). (C) lysed cyanobacterium with intact cells in cyanocyte; lysis apparently an artefact of fixation (x 17,000). (D) Cyanobacteria (Cy) and bacteria (Ba) in same vacuoles of cyanocyte (x 13,400). Cm: cytoplasmic membrane; Mc: mitochondrion; Mh: mesohyl; Nu: nucleoplasm; Sc: sponge cytoplasm; Th: thylakoid; Wm: cell wall membrane

largest proportion of the cyanobacteria occurred in the mesohyl as normal intact cells with occasional dividing cells. The cyanobacteria were particularly numerous in the ectosome of *J. stellifera*, and occupied a greater volume than either the sponge cells or the bacteria. Smaller populations existed in *N. irata*, and few cyanobacteria were seen in *P. heteroraphis*. In the small vacuoles there was evidence of lysis and digestion of cyanobacteria with the release of granular material (Fig. 3A), similar to the glycogen demonstrated by Vacelet (1971) in sponge cyanobacteria. Lysis of cyanobacteria in the mesohyl and large vacuoles was rarely observed. Such lysis appeared to be an artefact of fixation, since the cells had a regular appearance except where rupture of the cell had occurred (cf. Figs. 3A and C).

The large aggregates of cyanobacteria occurred in the vacuoles of specialized archeocytes for which the name "cyanocyte" is proposed. The vacuolated cyanocytes have the following characteristics: contain numerous intact cyanobacteria with division rarely evident (Figs. 1C, 2D); contain occasional bacteria (Fig. 3D); contain much less mesohyl collagen than the adjacent mesohyl; frequently have an opening as a result of the incomplete closure of the apposing vacuole pseudopodia. Cyanocytes were often observed in intimate contact with other mesohyl cells (Fig. 2E). The cyanocytes in *Jaspis stellifera* were larger and more numerous than those in the other sponges, and contained larger populations of cyanobacteria.

Two distinct types of cyanobacteria were observed. Those in *Pericharax heteroraphis* were small (Table 1), of irregular structure and density, and had a thylakoid with 1 or 1½ spirals (Fig. 1D). Those in *Jaspis stellifera* and *Neofibularia irata* were larger (Table 1), denser and more regular, and had a thylakoid with from 2 to 8 spirals (Figs. 2C, 3B). The number of spirals was proportional to the depth within the sponges, with the least number occurring in the outer, more-illuminated tissue (Vacelet, 1971). The cyanobacteria were usually surrounded by a distinct, capsule-like, clearing (Fig. 2F), which did not appear to be an artefact of fixation as it was not evident in neighbouring sponge cells (Fig. 2D, G). Capsules occur frequently in cyanobacteria (Wolk, 1973).

Bacteria

Bacteria were observed throughout all 4 sponges, but were concentrated around the inhalant canals in *Ircinia wistarii* (Fig. 4A) and were less numerous adjacent to cyanobacteria in the illuminated ectosome of *Jaspis stellifera* (Fig. 2G). The majority of bacteria were observed as free-living cells in the mesohyl, and a small number occurred in small sponge cell vacuoles where there was evidence of lysis and digestion (Fig. 4B). The bacteria, with few exceptions, had a cell wall structure and mode of division typical of Gram-negative bacteria (Wiebe and Chapman, 1968).

Few bacteria were observed in the mesohyl of *Pericharax heteroraphis*, and

Table 1. Morphological characteristics of bacteria and cyanobacteria from *Pericharax heteroraphis (Pe)*, *Jaspis stellifera (Ja)*, *Neofibularia irata (Ne)* and *Ircinia wistarii (Ir)*. Measurements and description obtained from electron micrographs. +: present, -: absent, RNP: ribonucleo-protein, DNA: deoxyribonucleic acid

Type	Occurrence	Width (μm)	Length (μm)	Cell wall (nm)	Capsule (nm)	Outer sheath	RNP cytoplasm	DNA nucleoplasm
Bacteria								
1	*Pe, Ja, Ne*	0.3 - 0.4	1.0 and +	25 - 45	-	-	dense, compact	medium, central
2	*Pe, Ja, Ne*	0.6 - 0.8	1.0 - 1.5	40 - 45	16 - 24	+, wide	medium, diffuse	thin, diffuse
3	*Pe, Ja, Ne*	0.6 - 0.8	1.0 - 1.5	40 - 45	15 - 25	-	medium, diffuse	thin, diffuse
4	*Ja*	0.7 - 0.9	1.2 - 1.4	150 - 300	-	-	dense, wide	dense, central
5	*Ja*	0.4 - 0.6	0.6 - 1.0	30 - 35	40 - 50	-	thin, diffuse	dense, scattered
P85, J69, G72	*Pe, Ja, Ne*	0.5 - 0.7	1.0 - 1.5	25	-	+/-	medium, diffuse	thin, scattered
I46	*Ir*	0.4 - 0.6	1.4 - 1.6	20 - 25	-	-	thin, scattered	thin, scattered
I78	*Ir*	0.4 - 0.5	0.6 - 0.8	25 - 30	-	-	medium, diffuse	medium, scattered
Cyanobacteria								
a	*Pe*	1.0	1.5	40	+/-	-	thylakoid: 1-1½ turns, irregular	dense, scattered
b	*Ja, Ne*	1.4	2.0	25 - 45	+	-	thylakoid: 2-8 turns, regular	dense, central

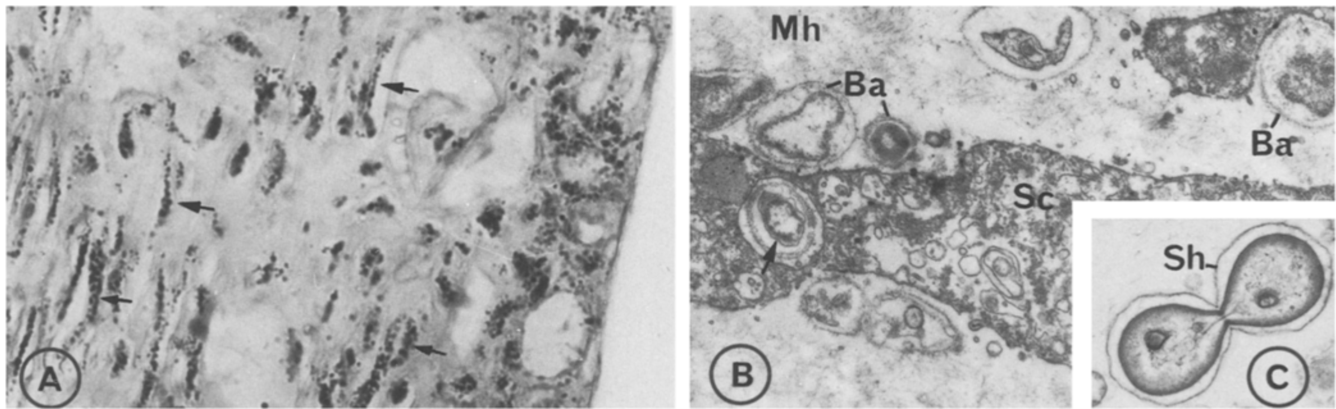

Fig. 4. *Ircinia wistarii.* (A) Inhalant ectosome with aggregates of bacteria in distinct tracts (arrowed) (x 480); (B) bacteria (Ba) adjacent to and inside sponge amoeboid cell – note cell in vacuole (arrowed) (x 13,400); (C) dividing sheathed bacterium in mesohyl, similar to but larger than Type 2 (x 15,600). Mh: mesohyl; Sc: sponge cytoplasm; Sh: sheath

these were predominantly Type 1 (Table 1; Fig. 2I). Bacteria were particularly numerous in *Jaspis stellifera,* and were estimated to constitute up to 40% of the mesohyl volume in parts of the endosome, far in excess of sponge cytoplasm. Many morphological types of bacteria were observed, but Types 1, 2 and 3 were predominant and in the relative proportions of 5:2:4 (Table 1; Figs. 2H, I, J). Among the rest, Types 4 and 5 were consistently observed (Figs. 2K, L). Types 2 and 3 were morphologically similar except for an outer sheath (Fig. 2J), which is not present in Type 3 cells. The sheath appears to be the bounding layer of a bacterial capsule, and in some instances it is barely visible (Fig. 2H). Type 4 cells were unusual in that they had a particularly wide cell wall. The bacterial populations in the mesohyl of *Neofibularia irata* were small, with Type 1 being the most conspicuous. Few Type 2 cells were seen and Type 3 cells were rarely observed. The numerous bacteria in *Ircinia wistarii* occurred either as distinct tracts in the ectosome or closely associated with sponge cells in the endosome (Fig. 4B), where they had an involuted appearance. Many morphological types were evident which, with the exception of some cells similar to Type 2 (Fig. 4C), did not resemble those seen in the other sponges. A large proportion of the bacteria had external sheaths and contained mesosomes (Fig. 4C).

The bacterial strains selected from the major cluster groups isolated from *Pericharax heteroraphis* (P85), *Jaspis stellifera* (J69) and *Neofibularia irata* (G72) were shown to be morphologically similar (Table 1; Fig. 5A, B and C). These bacteria have a Gram-negative morphology with a planar cell wall membrane, but some cells in the preparations had an irregularly undulating membrane (Fig. 5C). These bacteria produce sticky-mucoid colonies in culture, and this was evident in the capsule-like structures around many cells (Fig. 5B). Mesosomes were not observed in these preparations.

The two bacterial strains selected from the principal cluster groups in *Ircinia wistarii* (I46 and I78) resembled typical Gram-negative bacteria (Table 1; Fig. 5D, E).

Discussion

The sponge/microorganism associations appeared constant, with no evidence of either seasonal or intraspecific variations. The majority of the symbionts were free-living in the mesohyl without evidence of close interaction with the host sponge cells. However, the cyanocyte situation is indicative of a close association between cyanobacteria and certain sponge cells. This association is similar to those described by Smith *et al.* (1969) in mutualistic symbioses in which the host regulates the reproduction and metabolism of the symbionts resulting in a translocation of nutrient. Few dividing cyanobacteria were observed in the cyanocytes, suggestive of some host control, and the cyanocytes were often in close contact with other cells indicating a possible exchange of nutrient. Translocation of nutrient cannot be interpreted from ultrastructural evidence alone, as claimed by Sarà (1971),

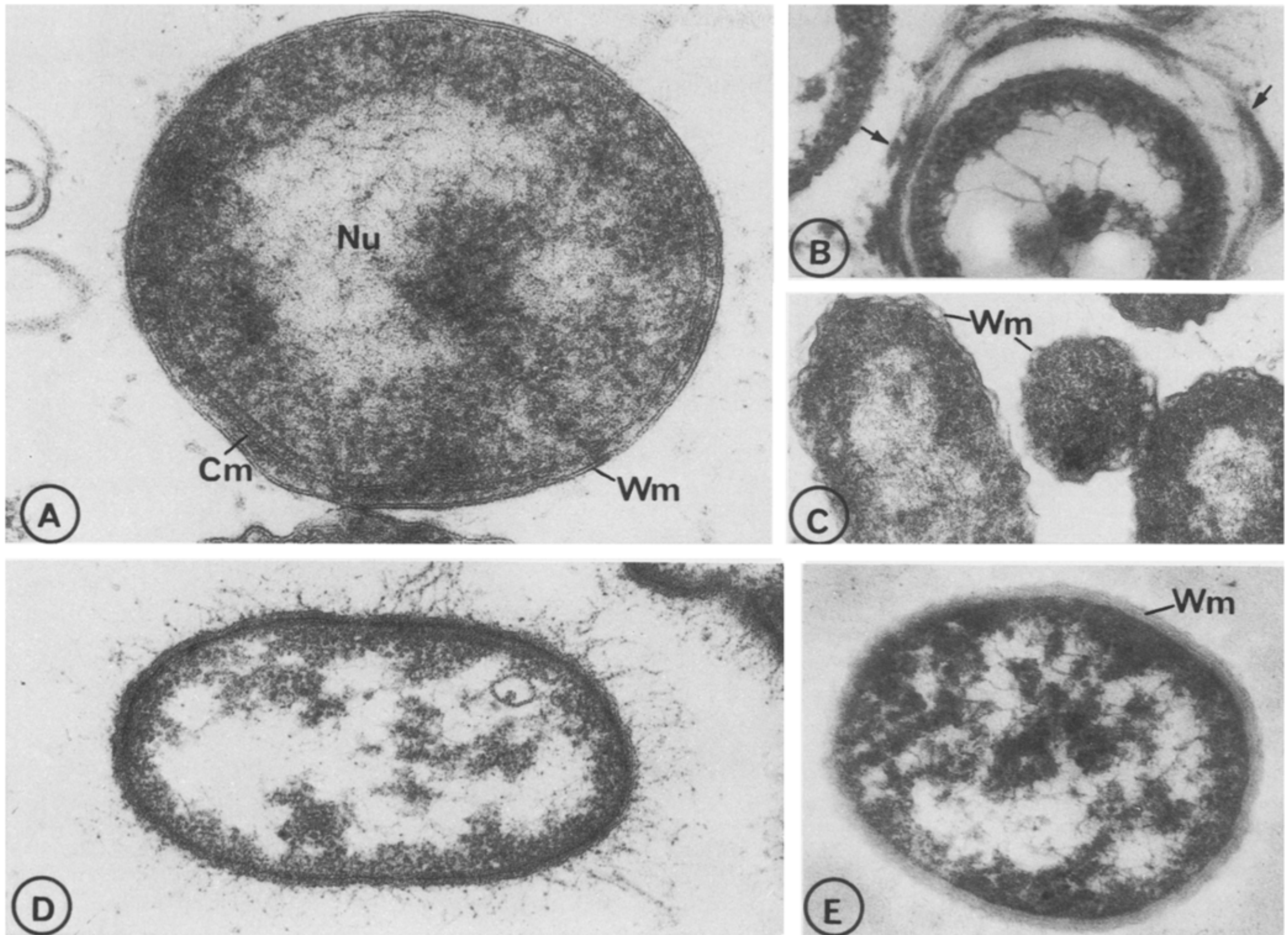

Fig. 5. Cultured bacterial strains (A) Strain J69: cross section of a bacterium stained prior to embedding with 1% uranyl acetate to emphasize the cell wall (x 104,000); (B) Strain J69: sheath-like structure (arrowed) formed around a bacterial cell (x 61,400); (C) Strain G72: cells with irregularly undulating cell wall membranes (Wm) adjacent to others with planar cell walls (x 51,000); (D) Strain I46: rod-shaped cell with typical Gram-negative cell wall (x 77,400); (E) Strain I78: section through a coccobacillus showing wide, pale-staining cell wall and irregular cytoplasm (x 75,100). Cm: cytoplasmic membrane; Nu: nucleoplasm

although Vacelet (1971) demonstrated histochemically the presence of glycogen in and around cyanobacteria associated with sponges.

Sponges efficiently remove microorganisms from the ambient water (Reiswig, 1971; Wilkinson, 1978a) and digest them intracellularly; however, few bacteria or cyanobacteria were observed being digested in mesohyl archeocytes. Extracellular lysis of symbionts was rarely observed and was almost certainly autolysis or an artefact of fixation. Thus, the digestion of microbial symbionts is not considered to yield a significant amount of energy to the host sponge. The claim by Sarà (1971) that extracellular lysis of cyanobacteria, observed in preparations with many osmotically disrupted sponge cells, contributed significant energy to some sponges is refuted.

Cyanobacteria were observed only in illuminated sponge tissue. Those in *Jaspis stellifera* and *Neofibularia irata* (Figs. 2C, 3B, respectively) are morphologically similar to those described by Vacelet (1971) in size (1.4 µm x 2.0 µm versus 1.3 µm x 2.5 µm); cell wall thickness (25 to 45 nm versus 35 to 55 nm); and the number of turns in the thylakoid (2 to 8 versus 1 to 4). The cyanobacteria from *Jaspis stellifera* and *Neofibularia irata* have a three-layered cell wall, with an inner cytoplasmic membrane to which is attached the thylakoid membrane, a peptidoglycan-like layer and an outer cell

wall membrane, often undulating. Vacelet (1971) described several additional layers which may have resulted from the staining of sponge material around cyanobacterial capsules (Wolk, 1973). The cyanobacterium in *Pericharax heteroraphis* differs from those described in other sponges in size and the nature of the thylakoid and cytoplasm (Table 1), and appears to be a novel symbiont. Comparisons cannot be made with the cyanobacteria from other Mediterranean sponges which Sarà (1971) inadequately described and identified as species of the genus *Aphanocapsa*.

The populations of bacteria observed in the sponges are proportionally similar to those counted previously (Wilkinson, 1978a). Type 2 and 3 cells are morphologically similar, with the only apparent difference being an irregular sheath of probable sponge origin around a bacterial capsule (Fig. 2H). Together, Types 2 and 3 constitute approximately 55% of the bacteria seen in *Jaspis stellifera*, but they were not conspicuous in the other sponges. Type 1 cells were the other prominent type, representing approximately 45% of the bacteria seen in *J. stellifera* and being predominant in *Pericharax heteroraphis* and *Neofibularia irata*. Only one distinct group was obtained during a numerical analysis of bacteria isolated from *J. stellifera*. Although similarities are evident between the bacteria observed in the sponges and the cultured strains (Table 1), ultrastructural examinations alone are inadequate for a definitive comparison.

The bacteria in *Ircinia wistarii* are concentrated around the inhalant regions, and consist of many morphologically different cells, unlike the populations in the other three sponges. No dominant type was obvious, although bacteria similar to the cultured strains (I46 and I78) were evident. Bacteria isolated from this sponge were classified into many groups of non-specific aerobes (Wilkinson, 1978b).

Some of the bacteria observed resemble those described by Vacelet (1975). Type 2 cells resemble the Type D cells of Vacelet, although he interpreted the outer sheath as being an external membrane. Type 4 cells are similar to Vacelet's Type E in having a dense layer between the cytoplasmic and cell wall membranes. Type 1 cells resemble the filamentous cells that Vacelet (1970) observed in the nucleus of *Verongia* spp. cells. Thus, there are some similarities in the bacterial populations of sponges from different regions but the proportions observed are often different, e.g. Type 4 cells are rare in *Jaspis stellifera* whereas Vacelet (1975) reported that Type E cells were abundant in *Verongia* spp.

Bacteria and cyanobacteria coexist in the sponges (Figs. 1B, 2G), although there are more cyanobacteria than bacteria in the illuminated tissue due to the availability of light energy. The populations of these microorganisms were maintained within animals which obtain at least part of their nutrition from digesting microorganisms (Reiswig, 1971). The digestion of few bacteria and cyanobacteria by mesohyl cells resembled a clean-up operation of degenerate or excess cells. Thus, the question is raised: are sponges able to recognise their microbial symbionts or is recognition as food material and phagocytosis prevented by protective extracellular layers around the microorganisms? Selective recognition in sponges would appear precocious and imply the existence of immune-type systems, but such systems have been postulated in corals (Hildemann *et al.*, 1977).

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