



Morphological aspects of symbiosis

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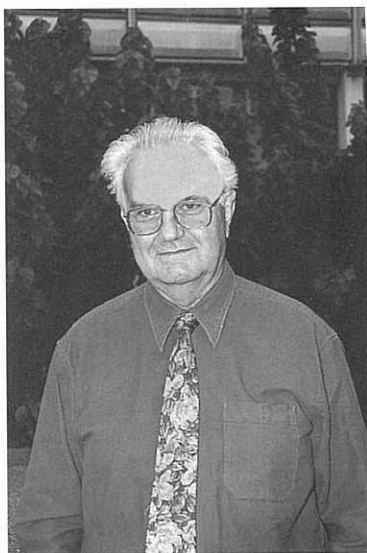
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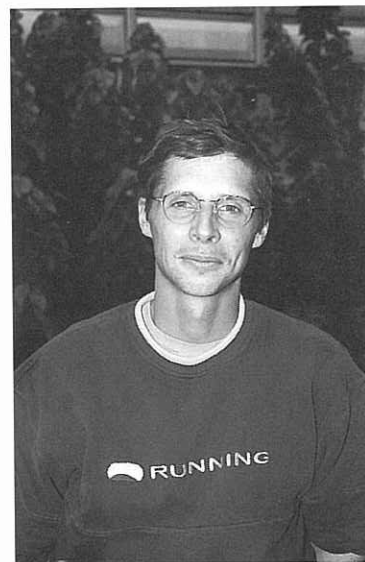
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MORPHOLOGICAL ASPECTS OF SYMBIOSIS

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1. Introduction and terminology

Most animals and numerous plants live in close association with microorganisms, and the concept of symbiosis has long been discussed (De Bary, 1879 - Smith, 1992 – Saffo, 1992 - Margulis, 1993 – Schenk, 1993 - Nardon and Nardon, 1998). In this chapter, we retain the simple definition of De Bary: "Symbiosis is the permanent association between two or more specifically distinct organisms, at least during a part of the life cycle." The typical parasitic and pathogenic interactions will not be included, because it is often difficult to distinguish between weakly aggressive and neutral associations. An example is the α -proteobacterium *Wolbachia*, often associated with insects and other invertebrates, and considered either as a parasite or as a neutral partner, according to Werren (1997). In general, one partner is bigger than the other(s) and is termed "the host", while the smaller partners are called the "symbiotes", or "symbionts". From an epistemological and historical point of view, "symbiote" is the correct term and not "symbiont" (Hertig *et al.*, 1937 – Steinhaus, 1949).

Symbiologists are sometimes not consistent, and the nomenclature changes according to authors. Here we propose to use the following terms to define different aspects of symbiosis:

Ecto or exosymbiosis: The partners remain external to each other, even if they are very intricately associated, as in lichens. In *ectosymbiosis* the smaller partner is an *ectosymbiote*.

Endosymbiosis: The smaller associates, *endosymbiotes*, are inside the host but remain extracellular. Most of the time *endosymbiotes* are in the digestive tract, or inside special organs.

Endocytobiosis: Intracellular symbiosis as defined by Schwemmler (1980). The *endocytobiotics* can be temporarily extracellular if there is a migration phenomenon, for instance to invade ovaries.

Symbiosome: Endocytobiotics are generally inside a host's vacuoles and the structure is termed a symbiosome (Ahn *et al.*, 1990). Symbiosomes must be considered as cell components. They contain one or more endocytobiotics (see the corresponding chapter in this book).

In endocytobiosis, most of the time, endocytobiotics are only harbored in special cells: *bacteriocytes* (inhabited with bacteria), *mycetocytes* (inhabited with yeast or fungi), *virocytes* (inhabited with viruses), *algocytes* (inhabited with algae or a plants).

These cells are often aggregated and form organs called bacteriomes or mycetomes. Special organs for viruses are not yet known. Protozoa are often intracellular at some stages of their life cycles, notably *Sporozoa* and *Microspora* (Bannister, 1979). But they do not form a special organ and are generally pathogenic. Nevertheless their host cells could be called "protistocytes".

In this chapter, we first show that symbiosis is not a biological curiosity but a common phenomenon in all biotopes on the earth. In the last years we observed a renewal of interest in this field. Second, we demonstrate the fact that symbiosis is a powerful factor for biodiversity, at the morphological level, inducing or favouring the formation of different structures playing a role in adaptation. As Moran and Telang (1998) stated "Endosymbiosis appears to facilitate diversification within ecological niches that would otherwise be inadequate". Third, comparing different models, we try to put forward, beyond the morphological diversity, the presence of common features, laws, or mechanisms implicated in host-symbiote relationships. In this short review it is impossible to present all the models, and the reader is referred to recent publications for further details: Jeon (1983) - Werner (1992) - Margulis (1993) - Nardon and Grenier (1993) - Sato *et al.* (1993) - Douglas (1994) - Nardon and Nardon (1998) - Seckbach (1999) - Moran (2000).

2. The partners in ectosymbiosis

A large number of ectosymbiotic associations of various types have been described, involving protozoa, algae, fungi, plants, and animals.

2.1. ECTOSYMBIOSES INVOLVING FUNGI

2.1.1. Lichens

Lichenologists (Franck and De Bary) developed the concept of symbiosis in the 19th century. Lichen is an association between a fungus (= mycobiont) according to Werner (1992) and an alga (= photobiont) or a cyanobacterium. The two partners show different degrees of intimacy. In the less complicated lichens, like *Collema*, the alga and fungus are simply disposed side by side. In the most intricate lichens, the fungus may develop special devices, the haustoria, to penetrate the gonidia. In all cases the association constitutes the thallus (see Honegger, 1991).

The fungus generates spores that germinate and find an alga to form new lichen. *In vitro* assays show that the fungus alone may form a thallus, but, to be perfect, the presence of alga is necessary. What is intriguing is the fact that symbiosis creates a new structure, that is perfectly stable from one generation to the next, genetically and epigenetically controlled (the role of alga), probably thanks to signal exchanges between both partners.

We put forward two facts: First, the thallus, for a given association, has always the same morphology and cytology (Des Abbayes, 1963 - Werner, 1992). We can distinguish four types of lichens: crustose, leprose, foliose and fruticose. The crustose type (*Collema pulposum*) is flat, tightly fixed to the substrate, and with a homogeneous structure. The leprose type is a primitive crustose type with granular structure (*Lepraria*). The foliose type (*Parmelia*) presents four distinct layers: the upper cortex,

the photobiont layer, the mycobiont layer, and the lower cortex with the rhizines, the organs of fixation to the substrate. The fruticose type is represented by *Usnea longissima* showing a radial symmetry. Pendant on trees, it is the largest lichen with a length of several metres.

The second is the fact that in lichens both partners are modified (Galun, 1990). For instance, filamentous cyanobacteria in *Scytonema* are changed to a unicellular form without heterocytes (specialized cells fixing atmospheric nitrogen). There are also modifications in the cell wall (in *Trebouxia*). The plasticity of cyanobacteria has been well studied (even if the mechanism is yet unknown), and they are involved in numerous symbioses (see Bergman *et al.*, 1999).

2.1.2. Ectomycorrhizae

Ectomycorrhizae represent symbiotic associations between fungi and root plants, the fungus mycelium coating young roots. Hyphae can also penetrate into the roots, but they are always extracellular (with few exceptions), forming the Hartig net (Werner, 1992). The structure of ectomycorrhiza is essentially determined by the fungal species rather than by the host plant. The root cap and the meristem remain uninfected. In some plants, like *Pinus radiata*, the ectomycorrhizae do not induce morphological changes in the root. But in other trees, like *Betula*, modifications appear and establish a particularly close contact between partners. The number of root cells remain unchanged, but they swell, giving rise to a club-shaped form to the swollen root tips.

2.1.3. Other type of fungi/plants associations

A great number of plants have pathogenic and symbiotic relations with fungi (see Werner, 1992 – Anderson *et al.*, 1984 – Pirozynski and Hawksworth, 1988). An interesting model is that of grasses associated with Clavicipitaceous fungal endophytes. The hyphae penetrate grass tissues, and, depending on the species, become either a parasite, as *Claviceps*, which sterilizes the grass ovaries, or a symbiote, as *Balancia*, which produces alkaloids protecting the grass against herbivorous animals. In *Epichloe* infection, a stroma is formed with plant and fungal tissues. The mesophyll cells may be hypertrophied and then the stroma appears as an enlargement of the stalk.

2.1.4. Fungi/Insects ectosymbiosis

Fungi are not only frequently associated with plants but also with animals, especially in insects: Thus, some ants, termites, Coleoptera, and wood-wasps cultivate fungi and feed their larvae.

Despite the non-permanent contact between an insect and the fungus, some special devices can differentiate in the host. In the wood-boring Coleoptera (Scolytidae and Platypodidae) about 36 genera cultivate symbiotic fungi in galleries excavated by the mother (Francke-Grosmann, 1967 – Batra, 1967). The fungus is inseminated in the galleries by adult insects. What is curious is the fact that, in Scolytidae, the spores are transported inside depressions of the cuticle associated with setae (Levieux *et al.*, 1991), located on the elytra, on the mandibles or on the sides of pronotum. The Platypodidae have special fungus-carrying organs, the mycangia (Nakashima, 1975), which are cavities associated with glandular cells. They are present either in female or male, or in both sexes (Beaver, 1989; Haanstad and Norris, 1985).

Lymexilidae (Coleoptera) and wood-wasps (Hymenoptera) also possess mycangia. These mycangia are absent in non-symbiotic species, and they are considered as possible products of symbiosis. It is fantastic to imagine how these mycangia could have been formed, probably during coevolution of the partners.

Another case of ectosymbiosis with fungi is the association of leaf-cutting ants (*Acromyrmex*, *Atta*) with a cultured fungus: *Attamyces bromotificus* (Cherret *et al.*, 1989). This type of symbiosis is obligate for the two associates. The fungus is the food for larvae and adults. It has never been discovered outside attine nests. The queen transports the fungus in a special pocket when she establishes a new colony.

Other examples of ectosymbiosis are the fungus-growing termites (Noirot, 1980 – Wood and Thomas, 1989). In each nest of Macrotermitinae there is a comb on which grows the specific fungus *Termitomyces*. We also find a *Xylaria*, but only in small quantities. The comb is mainly built from fresh fecal material. What is curious is the characteristic form of the comb, unique to each species of termite.

2.2. ECTOSYMBIOSIS IN ANTS/PLANTS

In the tropical zones, several species of ants live in tight association with trees like *Acacia* or epiphytes (Jolivet, 1986). Some special devices can differentiate in host plants, as domacia [Figure 1]. Domacia are specific structures inhabited by ants when they are not at work. It is not known if these domacia were preexisting to ants or not, but they are a “symbiotic organ”, useful both to the tree (a water reserve) and to the insect (a shelter).

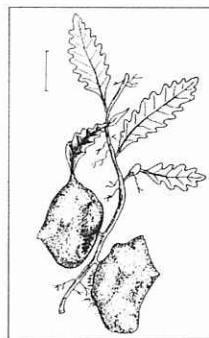


Figure 1. Domacia on the fern-ant epiphyte *Solenopteris* sp. The ants stay inside the modified rhizoma. Bar = 2 cm. (from Rauh 1955, with permission of *Annals Soc. Entomol. Fr.*)

2.3. ECTOSYMBIOSES INVOLVING PROTOZOAN OR ALGAE

In some biotopes such as benthos or tropical planktonic communities, the silica walls of diatoms are colonized by various ectosymbiotes or epibionts: cyanobacteria, dinoflagellates, ciliates and even diatoms (as *Nitzschia*). Furthermore some ciliate species also possess ectosymbiotes, like diatoms. We may also have dinoflagellates fixed upon foraminiferan. All combinations seem possible (Taylor, 1982). The specificity is not so great, and we may suppose weak interactions between host and ectosymbiotes.

In one particular biotope, the intestine of termites, and particularly in the paunch of the hindgut, some endosymbiotic protozoan, like *Joenia annectens*, are covered with bacterial ectosymbiotes, (Radek *et al.*, 1992 – 1996 ; see also Ohkuma in this book). *Mixotricha paradoxa* is a flagellate in the hindgut of *Mastodermes darwinensis*. It possesses a large number of bacterial ectosymbiotes which are small spirochetes (about 500,000) and less numerous large spirochetes. At the base of spirochetes, also attached to the host cell, are short rod-shaped bacteria (Cleveland and Grimstone, 1964 - in Margulis, 1993). It must be emphasized that the attachment of these ectosymbiotes is not random but they form regular rows and their undulations are coordinated, so that they play a crucial role in the protozoan motility. In a devescovinid flagellate from a Florida termite, two bacterial ectosymbiotes are also regularly attached on the surface (Tamm; 1980). Freeze-fracture and thin-section electron microscopy show that surface specializations in both partners occur at the junctional complexes. Rod bacteria lie in pockets of the eukaryotic membrane which are coated by dense material and contain numerous intramembrane particles. Fusiform bacteria are attached along ridges of the flagellate surface. The surface of the rod bacteria in contact with the medium bears a thick glycocalyx and flagella. We have an architecture. Who is the architect? The control of junction assembly by the cortical bacteria is probable, but how does it work? In *Joenia*, removal of the ectosymbiotes with antibiotics, shows that the specialized contact sites are present in the absence of bacteria. But nothing remains of the former attachment sites in axenic *Devescovina* cells. In this case, these sites are perhaps induced by bacterial contact. But there does not seem to exist a general mechanism for bacterial attachment in these symbiotic associations (Radek *et al.*, 1996).

2.4. ECTOSYMBIOSE IN INVERTEBRATES

The pompeii worm *Alvinella pompejana* is an Annelid living in deep-sea hydrothermal vents (Gaill *et al.*, 1987 – Cary *et al.*, 1997). The worms are in a tube, and do not seem to have intracellular bacteria, but the teguments of the worm are covered with microorganisms of various types belonging to the ϵ -proteobacteria group. Four types are visible on the dorsal part: (a) rod-shaped bacteria linked to the cuticle of the worm by thin filaments; (b) small spiral-curved bacteria, (c) bacteria with appendages, and (d) filamentous bacteria, which are most abundant. In *A. pompejana* the role of the bacterial community has not yet been elucidated and no specific morphological changes have been described.

3. The partners in endosymbiosis

The intestinal symbioses are probably the most widespread endosymbioses in animals. We may also have associations in special organs and diverse others forms of symbiosis with prokaryotes or eukaryotes. The endosymbiotes remain extracellular.

3.1. SYMBIOSES IN THE INTESTINE

We shall limit our discussion to termites and to mammals that are phylogenetically very distinct.

3.1.1. Mammals

From the point of view of symbiosis, we can distinguish two groups of mammals: monogastrics and ruminants.

Monogastric mammals. They are nonruminants, such as humans and rodents. According to Savage (1977), a normal human organism is composed of over 10^{14} cells, of which only about 10 % being animal cells. What we call a human, at least from a zoological point of view, is really a consortium of more than 300 different species. The associated microorganisms are principally bacteria living in the gastrointestinal tract (Ducluzeau and Raibaud, 1979), or yeasts. We cannot describe this complex microecosystem in detail here, but we emphasize that it is not a uniform biotope. The physical, chemical and cytological characteristics change all along the digestive tract, and the microbiota also changes: in mouse, short bacilli (1-2 μm) are attached on the stomacal epithelium, whereas in the duodeum there are filamentous (more than 8 μm long) bacteria. Different niches exist and 40 % of the wet weight of fresh feces of the mouse is from bacteria.

Endosymbiotes are in the lumen or in the alimentary bolus, or are attached to the gut epithelium. Some long bacteria are unique to the small bowel of mammals and have never been found elsewhere. Comparison of holoxenic normal intestine with axenic ones (deprived of endosymbiotes) shows that microorganisms can modify the appearance of the digestive tract. In the ileum of axenic rats, enterocytes have microvilli longer than those in axenic rats. Furthermore the intestinal wall of an axenic rat is thinner and weaker than the normal wall, and the number of mucous cells is greater in the xenic epithelium. Another difference concerns the Peyer organs, which are atrophied in the absence of symbiotes. These organ play an important role in immunodefence of an organism (secretion of immunoglobulines).

Ruminants mammals. They are polygastric, and the anterior intestinal tract is greatly modified to facilitate symbiosis [figure 2]. The principal compartment is the rumen. It reaches 250 l in the cow and represents 70-75 % of all the intestinal volume. The rumen is probably the most complex of all the known biotopes with a temperature of 40°C, pH 6-7 and a redox potential of -350 mV under anaerobiosis conditions. The bacterial endosymbiote population represents more than 200 species, among which about 30 are specific to the rumen. Most are strict anaerobic bacteria and are attached to the mucous epithelium and the vegetal particles. Their number is estimated to be 10^{10} cells/ml of rumen content. Other endosymbiotes are protozoa, essentially ciliates (10^4 to 10^6 cells/ml) which can ingest solid particles. Finally fungi are less numerous (10^3 to 10^7 spores/ml) (Hespell *et al.*, 1997).

3.1.2. Termites

All the termite species are not associated with fungi. Most of them live in symbiosis with bacteria and/or protozoa, located in their digestive tract. More recently, in lower termites, yeasts have also been found (Prillinger *et al.*, 1996). The hindgut is well developed and forms a paunch containing both bacteria and protozoa in the lower termites, which are wood-eating insects. But only bacteria are found in the higher termites (*Termitidae*), which are humivorous (Noirot, 1992). The density of symbiotic flagellates ranges from 10^3 to 10^7 cells per ml of gut fluid (To *et al.*, 1980) but it may reach 10^{10} /ml (Breznak, 1982-1984). These bacteria are either free in the gut lumen, or attached to the alimentary bolus or the gut wall, or associated with flagellates (see

Mixotricha). In this book, Ohkuma describes several methanogen species; they show a distinct spatial distribution in the gut. In the termite *Procubitermes aburiensis*, bacteria of the hindgut are attached to specific cuticular spines elaborated from the gut wall. Each spine is linked to a specialized root cell which exhibits a bundle of parallel microtubules extending from the apex to the base of the cell (Bignell *et al.*, 1980). It can be assimilated to a symbiotic organ, but we do not know how it has been formed.

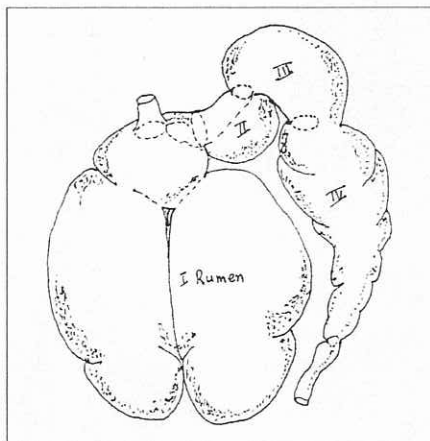


Figure 2. Anterior intestine of a ruminant. II: reticulum, III: omasum, IV: abomasum (the primitive stomach).

3.2. LUMINOUS ORGANS

The luminous organs are present in various animals: marine fishes, squids, crustaceans, nematodes and pyrosomes. The luminescence is due to the presence of bacteria (at least 10^7 cells/ml) which remain extracellular. We have chosen the squid model, *Euprymna scolopes*, colonized with highly luminous and specific bacteria: *Vibrio fischeri* (McFall-Ngai and Ruby, 1991, 1998). As described by the authors, "This symbiosis offers unique opportunities to understand the processes underlying the colonization of animal epithelias by benign bacteria".

The complex light-emitting organ is located in the center of the mantle cavity. In the adult *Euprymna* the organ is composed of two lobes with three distinct epithelium-lined crypts that house the *Vibrio*. As an accessory tissue there is a thick reflector which directs luminescence ventrally (Ruby, 1996).

The *Vibrio* is not heritable and the infection from the environment must be repeated for each generation. A mechanism of recognition is probably present to ensure the specificity of the association. At hatching, the organ bears two lateral pairs of appendages that facilitate the capture of *Vibrio* (Doi and McFall-Ngai, 1995). The crypts are connected by a pore to the surface of the organ. Bacteria enter the pores and reach the crypts where they are first attached to microvilli. They multiply and finally fill the space entirely (McFall-Ngai, 1999). Each day, at dawn, the host expels 90-95 % of its bacteria in the seawater. Being established, symbiosis induces modifications in both

the host and the endosymbiote (McFall-Ngai, 1999). The latter undergoes several changes in its morphology and physiology. Between 12 and 24 h after the initiation of symbiosis, the *Vibrio* loses the polar flagella characteristic of free *Vibrio* in the surrounding seawater. Furthermore, the endosymbiote decreases about 8 fold in volume and is more fluorescent.

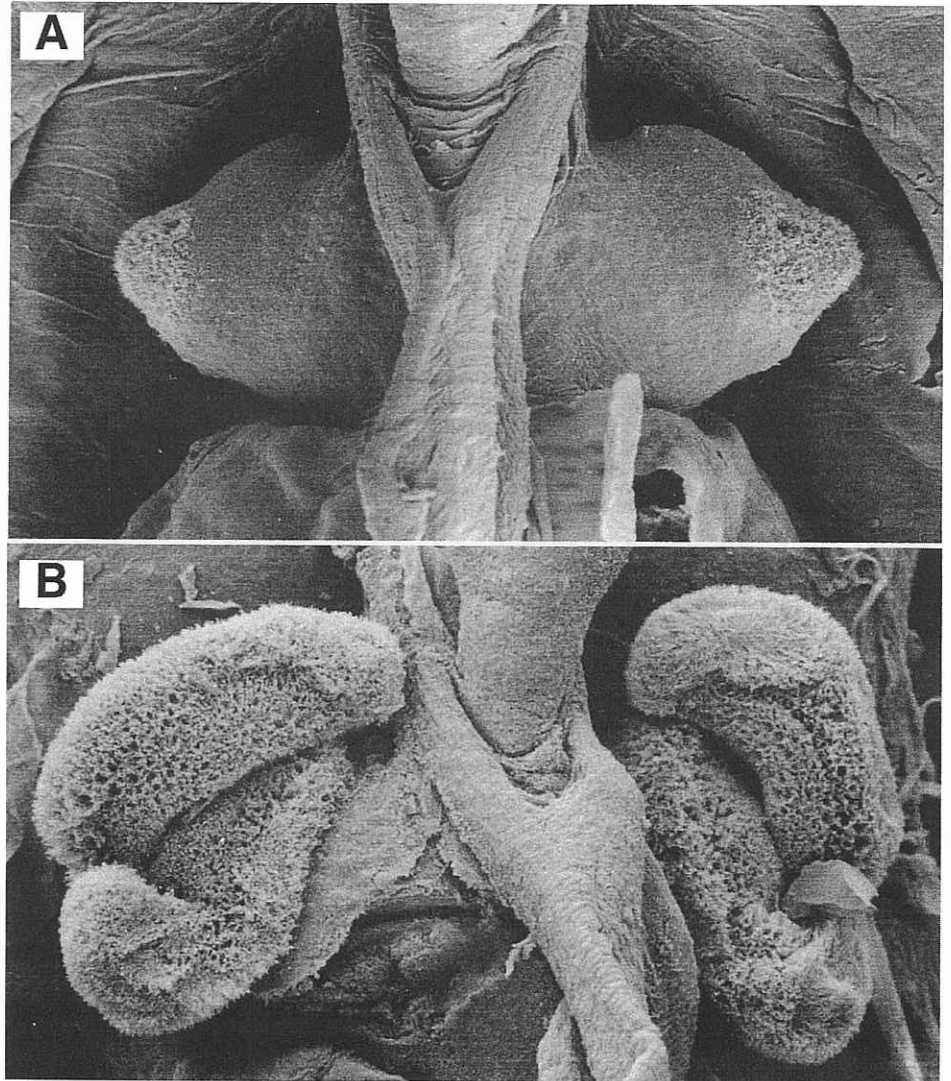


Figure 3. light organ of the squid *Euprymna scolopes*. A: ciliated and microvillous field on each lateral face of the light organ in 4-days old symbiotic animals. B: the same at hatching in absence of *Vibrio*.

The squid also shows important changes in the light organ, triggered by the bacteria. The structures elaborated before infection regress over a period of 4 days if the colonization is successful. This is particularly spectacular for the lateral appendages [figures 3A and 3B]. In uninfected squids these structures are retained. We have here an example which strongly suggests that symbiotes interfere with the normal program of development of a juvenile host. Only 12 hours of contact is necessary to induce cell death and tissue regressions of the external ciliated epithelium (McFall-Ngai, 1998). The epithelial cells in the crypts do not undergo cell death but exhibit a fourfold increase in volume over the first few days after colonization. The microvillar density also increases fourfold.

3.3. OTHER TYPES OF ENDOSYMBIOSIS

Endosymbiosis in plant does not seem to be a general rule, but in animals no invertebrate taxon appears to be entirely symbiote-free. For instance, symbioses between eukaryotes and bacteria are known in protozoans, sponges, cnidarians, nematodes, turbellarians, vestimentifera, molluscs, and arthropods. Here, we shall consider only the insect galls of plants, the echinoderm and sponge endosymbioses.

3.3.1. Insect galls

Some mite species induce the formation of plant galls but most of the time they are pathogenic, whereas insect galls are generally tolerated by the plants, if they are not too numerous. Among the gall-forming insects are cynipid Hymenoptera and cecidomyiid Diptera. The larvae are inside the gall, whose morphology is specific to the insect (Folliot, 1977) [figure 4]. Some aphids are also gall-forming insects. They live inside the gall whose morphology is specific: coral-shaped for *Tuberaphis taiwana* (Aoki and Kurosu, 1998), or as a spiral pouch for aphids living on the petiole of poplar leaves. The mechanism of gall induction is not yet fully elucidated; it is a fascinating problem of morphogenesis. In *Quercus* alone, more than 800 galls are known. The plant shows hyperplasy, hypertrophy and dedifferentiation of cells (Cambar, 1953 – Forrest, 1987 – Pirozynski, 1991).

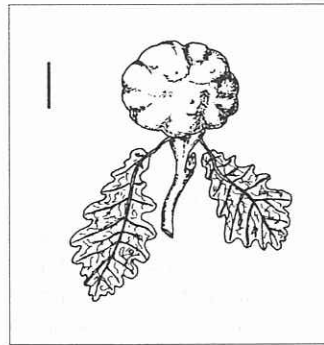


Figure 4. A gall induced on oak tree by cynipid larvae (*Biorhiza pallida*). Bar = 1 cm (from Folliot, 1977, with permission of *Annals Soc. Entomol. Fr.*).

3.3.2. Echinoderms

A study of Echinoderms in New Zealand and in British Isles, showed that among samples of 38 and 88 species, 17 and 63 species, respectively, harbored Gram-negative subcuticular bacteria (SCB). Their number is very important: about 10^9 cells/g ash-free dry weight of host tissue (Kelly *et al.*, 1995 – Kelly and McKenzie, 1995). The SCB can be classified into two major morphological types. One type lacks capsules and is usually spiral. It is most abundant. The other kind is rod-shaped, with capsules or vacuoles. The two types can be found together in the same host of some species.

3.3.3. Sponges

Some sponges are composed of 21% of animal cells, 38% of extracellular bacteria and 41% of intercellular substances (Bertrand and Vacelet, 1971). The study of symbiotic extracellular bacteria in four species of the sponge genus *Plakina* shows that the number and composition of bacterial types allow one to discriminate between species, and the pattern of distribution of bacteria may help to characterize the genus *Plakina*. The abundant bacteria are often grouped in zona devoid of collagen. Eight morphological types are described (Muricy *et al.*, 1999). Microorganisms living with sponges are diverse: heterotrophic bacteria, cyanobacteria, zoochlorella, diatoms and even fungi. Specialized organs are not formed, despite the fact that these associations are probably very ancient. Furthermore, sponges can also possess endocytobionts located in bacteriocytes (Vacelet, 1970). This "symbiocosm" is therefore very complex.

4. The partners in endocytobiosis

In the preceding sections concerning ectosymbiosis and endosymbiosis, we tried to present evidence for both the biodiversity of symbiosis and the convergent phenomena. Endocytobiosis is more widespread and more diverse, only a few models will be described. Here again we will describe common processes at the cytological level, or the possible other great differences. Among the interesting traits to analyze, we have the nature of the partners, the location of endocytobionts at cellular and organismic levels, their variability, their destruction, the structure of hosting organs (bacteriocytes, mycetocytes), and more generally all morphological consequences of symbiosis.

4.1. PROTOZOAN AS HOSTS

4.1.1. Bacteria

Most Protozoa live in symbiosis with bacteria of algae. *Amoeba proteus* is a wonderful model where the establishment of an endocytobiosis has been studied (Jeon, 1995). The amoeba was infected by pathogenic γ -proteobacteria in the first step, but, along the evolution of the association, the same pathogenic bacterium became not only harmless, but obligate for the amoeba, and this occurred during only one year of observation (Jeon, 1987). Therefore endocytobiosis is not necessarily a very old association, despite the fact that the bacteria appear to be very well integrated to the physiology of the host cell. About 42,000 bacteria are enclosed within host-generated vesicles, the symbiosomes (see corresponding chapter) (Ahn and Jeon, 1979). In this model, the physiological influence of endocytobionts is crucial (Jeon, 1983), but the morphological

changes are very discrete (Jeon and Lorch, 1967), perhaps because the symbiosis is very recent. The infected amoebae become smaller in size and yellowish in color.

The ciliate hypotrich *Euplotes* may have numerous endocytobiontes, harmful, neutral or beneficial. *Omikron* is a Gram-negative, obligate bacterium. The curved bacilli are spread into the cytoplasm (Heckmann, 1983 – Görtz and Brügge, 1998). Another ciliate, *Cyclidium pascuatum* exhibits a unique tripartite structure consisting of hydrogenosomes mixed with methanogenic archaeobacteria and eubacteria (Esteban *et al.*, 1993). This structure is stable, indicating that the ciliate is an anaerobic consortium of three different functional partners.

In the above species and in many others, symbiotic bacteria are located in the cytoplasm, generally in symbiosomes. But, in other examples, curiously, the endocytobionte invades the nucleus. Many are parasites, but *Holospora obtusa*, which belongs to the α sub-group of proteobacteria, is rather neutral for its host, the ciliate *Paramecium caudatum*. In the host macronucleus the symbionte is not surrounded by a membrane. It does not infect the micronucleus, and has a peculiar life-cycle (Görtz, 1983 – Dohra and Fujishima, 1993 – Kawai and Fujishima, 2000). The infectious form (13 μm long) penetrates into the ciliate cytoplasm by ingestion in a digestive vacuole. Then, the bacterium leaves the vacuole and is transported within the macronucleus for *H. obtusa*, or the micronucleus for *H. elegans* (Maier *et al.*, 1990), where they divide to be transformed in short bacilli (1.2 μm long). The latter no longer divide and elongate to differentiate into the infectious long form. Therefore we note the great morphological plasticity of these endocytobiontes.

The colonization of nuclei is also observed in other protozoan species. Görtz (1983-1986) proposes the term “endonucleobiosis” to designate this fact. The invaders are not all bacteria. In ciliate nuclei, parasitic algae may exist, or other protozoa like flagellates in *Stentor* where they induce a strong hypertrophy of the nucleus. More generally, endonuclear symbiontes cause morphological changes in infected nuclei: shape, size and location within the cell, but the effects depend on the host. Both endonuclear and cytoplasmic symbiontes may coexist, for instance in *Paramecium bursaria*.

4.1.2. Plastidic protozoa and algae symbioses

Plastidic protozoa is the name proposed for protozoa harboring functional plastids (=chloroplasts) in their cytoplasm (Laval-Peuto, 1992). This phenomenon is widespread and the plastids come from various classes of algae. In foraminifera they mainly arise from diatoms or chlorophytes. In the cytoplasm, symbiotic plastids are located at the cell periphery and their number varies. In the large oligotrich ciliates there are several hundred plastids per cell (more than 300 in *Tontonia appendiculariformis*). Three membranes generally enclose the plastids. The outer one, or periplastidial, is thought to be a product of the ciliate-host, which protects the plastids from digestion.

The association between the ciliate *Mesodinium rubrum* and chloroplasts of Cryptomonad is one of the most integrated symbioses. Each ciliate harbors 10 to 100 chloroplasts responsible for the maroon color. It also exhibits some modifications, interpreted as adaptations to the presence of the chloroplasts: the oral cone is reduced, the “mouth” is vestigial and the oral tentacles are lacking, as compared to the closely related non-symbiotic species. Furthermore, the ciliate swims with the oral end posterior and has a phototactic behavior (Taylor, 1982).

We have to distinguish between the presence of plastids, always limited in time, and true symbiosis with algae. Algae are able to reproduce and to be maintained in the strain.

Paramecium bursaria normally live symbiotically with several hundred intracellular *Chlorella* (Karakashian, 1975). They divide and are hereditary. The algae are within individual vacuoles. They divide at a rate always compatible with the *Paramecium* growth.

Some algal endocytobionts show little or no apparent morphological adaptations to their associations. But others exhibit modifications from their close relatives, such as reduction of wall structure and loss of flagella (in Foraminifera).

4.2. MARINE AND FRESHWATER INVERTEBRATES AS HOSTS

4.2.1. Prokaryotes as endocytobionts

Sponges. In the sponges a great number of bacterial endosymbionts are present, outside the cells (see above). But there are also bacteriocytes with endocytobionts as in *Verongia* (Vacelet, 1970). These bacteriocytes are large cells (10 to 32 μm in diameter) compared to other cells of the sponge (5 to 13 μm). The number of bacteriocytes is variable. They are not localized anywhere, but preferably near the surface of the animal and esculum. In another sponge, *Petrosia ficiformis*, cytobionts are in large membrane-bounded vacuoles. Several types of microorganisms are seen (Bigliardi *et al.*, 1993). In a symbiosis with cyanobacteria, Sara *et al.* (1998) show that the sponge presents changes in its morphology due to the cyanobacterium: larger size, modified shape and surface skeleton and decreasing number of pores. Perhaps these changes result from a better adaptation to photosynthesis. Intracellular endocytobionts are present in some species (Vacelet, 1970).

Echinoderms. In echinoderms, we may have the same situation as in sponges: the presence of bacteriocytes in supplement of the numerous extracellular endosymbionts. An example is *Ophiocoma ballonsi* (Hophiurid) which possesses bacteriocytes within the connective tissue of the tube feet (Kelly *et al.*, 1995).

Bivalve molluscs. Bivalve molluscs living in deep-sea hydrothermal vents, possess endocytobiotic chemoautotrophic bacteria. The two principal models are Vesicomysid clams *Calyptogena* and the Mytilid *Bathymodiolus* (Nelson and Fisher, 1995). Such symbiosis is obligate. The bacteria are inside vacuoles which can be interconnected (Fiala-Médioni *et al.*, 1990). They are highly integrated and colonize the gill cells. A study of *Calyptogena magnifica* shows that most of the gill tissue is composed of bacteriocytes. The endocytobionts never invade all the cells but are located under the ciliated zone. The external surface of each bacteriocyte is fringed by well-developed microvilli. The Gram-negative bacteria are rod-shaped and often grouped in clusters limited by a double membrane (Fiala-Médioni and Métivier, 1986). A closer observation allows the distinction of two types of associations.

First, in the mytilid *Bathymodiolus thermophilus*, bacteria only colonize the apical part of cells, which are not modified in their basal area. Second, in Vesicomysids *Calyptogena magnifica*, *C. phaseoliformis* and *C. laubieri*, host cells are completely transformed into bacteriocytes. In both types lipid inclusions and numerous lysosomes are also present (Fiala-Médioni and Le Pennec, 1987).

The most remarkable characteristic of all mollusc species living on the sites of deep hydrothermal vents of the East Pacific Ridge is the well-developed gill which is the main organ of nutrition, via the endocytobiontes (Fiala-Médioni, 1988). This is particularly true for the vesicomid *Calyptogena magnifica*, which has a reduced intestinal tract and reduced labial palps, and the absence of gut transit of particles. Furthermore the bacteriocytes and gill cells possess abundant microvilli in contact with the surrounding environment. Concerning the mytilid *Bathymodiolus*, the intestine is functional, with a transit of particles. The labial palps are well developed, but microvilli are reserved to particular cells. In this species the role of bacteriocytes seems to be complete and not to replace the role of the gut.

Solemya borealis is a bivalve living in anoxic sediments and contains a high concentration of chemoautotrophic bacteria in the gill. They are Gram-negative and rod-shaped endocytobiontes. Bacteriocytes are confined to the region proximal to the ciliated edge of the gill, and are flanked by intercalary cells (symbiote free). The gill is extremely hypertrophied and lacks an intestine (Conway *et al.*, 1992). Despite the notable difference in the habitat, the symbiosis appears morphologically very close to that in *Calyptogena*.

Two mytilid species (Cavanaugh, 1993) harbor another type of endocytobiontes, Gram-negative coccoid to rod-shaped and metanotrophic. These bacteria contain stacked intracytoplasmic membrane arrays. They are engulfed in vacuoles surrounded by a bacterial membrane. The bacteriocytes are interspersed with symbiote-free intercalary cells.

Concerning the biodiversity of symbiosis, the comparison between lucinid bivalves *Linga pensylvanica* and *Codakia orbicularis* is interesting since they both have the same sulfur-oxidizing bacteria, and they both live in the same tropical sea-grass beds. However, they exhibit different structures: absence of granule-cells in *Linga* while they are abundant in *Codakia*; numerous lysosomes in bacteriocytes of *Linga*, which do not exist in *Codakia*; complexity of the intercalary cells in *Linga* and different locations within bacteriocytes. The latter groups only occupy in *Codakia* the most superficial one third of the lateral zone, while in *Linga* bacteriocytes are located all along the lateral zone, intermingled with intercalary cells (Gros *et al.*, 1996).

The gastropod *Ifremeria nautili* lives in hydrothermal vent and possesses symbiotic chemoautotrophic bacteria in bacteriocytes (30 to 50 μm long) which comprise the majority of cells in the long ctenidial filaments. Each endocytobionte is in a vacuole, all vacuoles seem to be interconnected. As in bivalves, the host's stomach is reduced in size. From these different examples, it seems that the reduced intestine is an adaptation to the environment and especially to the presence of endocytobiontes (Windoffer and Giere, 1997).

Vestimentiferan worm *Riftia pachyptila*. This vestimentiferan is probably the most extensively studied animals living in deep-sea vents and seeps. It is a long worm (2 m or more), and its anterior end, the obturacular region, has a highly vascularized organ, the branchial plume, which is equivalent to a gill. The remainder of the worm is protected by a thick walled tube. What is unusual about this worm is that it has no mouth, no gut and no anus. However, it possesses an organ, the trophosome, which accounts for about 16 % of the animal's wet weight and consists of bacteriocytes, associated cells and blood vessels (Cavanaugh *et al.*, 1981 – Nelson and Fisher, 1995). The trophosome is also characterized by important sulphur quantities (Truchet *et al.*, 1998). The density of

bacteria is 10^{10} - 10^{11} cells per gram. The properties of bacteriocytes change during the time of association with the bacteria that are finally lysed, liberating nutritive metabolites for the worm (Bosh and Grassé, 1984 a and b). Symbiosis has completely modified the structures, and therefore the modalities of nutrition, probably by a coadaptation process. This is one of the best examples of morphological innovation induced by symbiosis.

4.2.2. Algae or plastids as endocytobiontes

Molluscs. Several mollusc species harbor plastids or algae as endocytobiontes. The marine mollusc *Elysia viridis* lives in symbiosis with chloroplasts of the alga *Codium fragile*. These algae are located in digestive cells, in vacuoles bounded by a host membrane which persists throughout (Hawes, 1979).

Coelenterates. The best-studied species of Coelenterate is the freshwater *Hydra viridis* (Muscantine *et al.*, 1975 a and b – Muscantine and McNeil, 1989 – Rahat, 1990, 1992). In nature they harbor endocellular algae (*Chlorella*) inside digestive cells, the cause of their green coloration. The brown hydra has no algae. When symbiotic, each digestive cell harbors about 12-20 *Chlorellae* in individual vacuoles. They are preferentially located at the base of host cells. The green algae are not directly captured by *Hydra*, but they have to be ingested by small crustaceans which are preys for *Hydra*. So, the algae are primarily filtered by these crustaceans which introduce the algae in the coelenteron where they are phagocytized by digestive cells of *Hydra*. Some algae are digested rapidly, or after some weeks, while others survive and reproduce. The reasons for such differences are not yet well understood. It seems that the regulation of algae is made at the endodermal level. Rahat (1990) introduced the notion of "preadaptation". Controlled by the host, *Chlorella* becomes heritable and a stable symbiosis is thus established. In general, in algal-invertebrate symbiosis, the wall of algae may be more or less modified. But in the *Hydra/Chlorella* symbiosis, no significant change has been observed.

4.3. PLANTS AS HOSTS

Despite their autotrophy, many plants are associated with bacteria or fungi, thus improving their nutrition.

4.3.1. Endomycorrhizae

In contrast to ectomycorrhizae, in endomycorrhizae hyphae of the symbiotic fungus penetrate into the root cells where they differentiate as vesicles and arbuscules mycorrhizae (VAM). This allows an increased surface of contact between the two partners (Meyer, 1967). When the VAM penetrate cells, the host plasmalemma is never ruptured and surrounds all branches of the arbuscule. There is no direct contact with the cytoplasm. The penetration of the fungus is marked by a considerable increase (23 fold) in the amount of cytoplasm and a regression of the vacuole typical of plant cells. The nucleus swells up and the chromatin is less condensed. Regression of the golgi apparatus is also observed. When the arbuscule degenerates, the cytoplasm decreases (Dexheimer and Gerard, 1990 – Berta *et al.*, 1990). It seems evident that these structures (vesicles and arbuscules) facilitate the exchange of metabolites. Finally, the VAM stimulate the production of adventitious roots (Scannerini and Bonfante-Fasolo, 1990).

4.3.2. *Rhizobium* symbioses

Rhizobium is a soil bacterium, capable of triggering a root modification in legumes, that leads to the formation of a nodule. This is the best-known model of symbiosis, where a lot of signals are exchanged between the partners, and the controlling genes have been identified. Not only *Rhizobium*, but also related bacteria of the soil, like *Bradyrhizobium* and *Azorhizobium* (*Rhizobia*), may elicit the formation of specialized organs in which symbiotic bacteria are able to convert atmospheric nitrogen into ammonia as a nitrogen source. Nodule formation is a multistep process (Van Rhijn and Vanderleyden, 1995 – Schauser *et al.*, 1999 – Oke and Long, 1999):

- *Rhizobia* move toward roots by positive chemotaxis to plant root exudates (amino acids, flavonoides...)
- Bacteria attach to the root surface, but only on young growing root hairs. The specificity is not strict, since *R. leguminosarum* may infect *Pisum*, *Vicia*, *Lathyrus* and *Lens* species. But *R. loti* is limited to *Lotus*. The adherence of *Rhizobium* is mediated by lectins on the host and special polysaccharide molecules present on the bacterial cell surface.
- The attachment of *Rhizobia* on young root hairs triggers characteristic deforming and curling via Nod Factors (lipochitin oligosaccharides), secreted by *Rhizobia*, under the control of nodulation genes.
- The young curled root hairs entrap bacteria in a pocket where a local lesion is formed by hydrolysis of the cell wall.
- *Rhizobium* enters roots by invagination of the plasma membrane.
- The bacterial infection induces a reaction of the plant, that forms a growing tube with cell wall material: the infection thread which is filled with growing bacteria surrounded by a mucopolysaccharide matrix.
- The infection thread carries bacteria from roots to the primordial nodules, that are formed in the root cortex where cell division is induced. The location of the nodule primordium depends on the plant species. It functions as a meristem.
- Cells differentiate and the primordium gives rise to the mature nodule, which consists of two types of tissues, the peripheral and central tissues. Four zones can be distinguished: meristem, invasion zone, infected zone and degenerative zone. The bacteria are released in the invasion zone, in cells which elongate and increase in size while bacteria proliferate.

Not only the plant but also the endocytobiontes are modified by symbiosis. When they are delivered in the nodule cells, inside vacuoles, they there are transformed into bacteroids, the form capable of fixing atmospheric nitrogen. From rod-shaped they become y-shaped bacteria. The volume increases 4 - 7 times, and they no longer divide. Numerous inclusions appear in bacteroids. Their location is perfectly controlled by the plant, and they are never found either in meristematic cells, or in the vascular zone. In this example the induction phenomena are spectacular, as in the luminous organ of the squid.

4.3.3. Other symbioses

Sesbania rostrata is a tropical legume plant which possesses nodules both on roots and on stems. The endocytobionte is a *Rhizobium* and the nitrogen fixation is very active.

Frankia is an actinomycete bacterium which can live in symbiosis with non-legume plants, where they induce the formation of nodules. They penetrate into curved root

hairs, and form infectious filaments, that penetrate some cells of the cortex where they become ramified and entangled. In *Alnus*, vesicles are differentiated. This first primordial nodule is transformed in mature nodule with the formation of new lateral root hairs.

The *Gunnera* are perennial rhizomatous plants with extremely long petioles (up to 2 m). They live in symbiosis with the cyanobacterium *Nostoc punctiforme* which is predominantly intracellular in all *Gunnera* species. They are located in the rhizomatous tissues (Meyer, 1967 – Osborne *et al.*, 1991). It seems that unusual red glands formed at the base of the first pair of cotyledons are the major site of entry for endocytobionts. Later, such red glands are formed at the base of each petiole.

TABLE 1. Examples of different types of endocytobiosis in insects.

Symbiotes	Host species	Location	References
Viruses			
S	<i>Drosophila simulans</i>	Epidermal cells	Louis, 1990
Polydnavirus	Parasitoid wasps	Oviduc calyx	Stoltz and Vinson, 1977
C	<i>Drosophila melanogaster</i>	Various tissues	DeBuron and Beckage, 1998
Bacteria			
Spiroplasma	<i>Antonia crawii</i>	Various tissues	Fukatsu and Nikoh, 2000
Flavobacterium	Cockroaches	Fat body	Bandi <i>et al.</i> , 1994
α -proteobacteria (<i>Wolbachia</i>)	<i>Callosobruchus chinensis</i>	Various tissues	Kondo <i>et al.</i> , 1999
"	<i>Nasonia</i>	Various tissues	Breeuwer and Werren, 1990
"	<i>Glossina sp.</i>	Various tissues	Chang <i>et al.</i> , 2000
γ - and β -proteobacteria	mealybugs	Bacteriome	Louis, 1967; Fukatsu and Nikoh, 2000
γ -proteobacteria	<i>Sitophilus</i>	Bacteriome around anterior gut	Nardon, 1971
"	<i>Camponotus</i>	Bacteriocytes in gut epithelium	Schröder <i>et al.</i> , 1996
"	<i>Cimex lectularius</i> (P)	2 bacteriomes, fat body	(a), Hypsa and Aksoy, 1997
"	Psyllid (P)	1 central bacteriome	Thao <i>et al.</i> , 2000
"	<i>Glossina</i> (P)	Bacteriome around ant. gut	Aksoy <i>et al.</i> , 2000
"	<i>Glossina</i> (S)	Midgut cells	Aksoy <i>et al.</i> , 2000
"	<i>Acyrtosiphon pisum</i> (P) (S)	Dispersed bacteriocytes in body cavity	Baumann <i>et al.</i> , 1997
Unknown bacteria			
"	<i>Euscelis plebejus</i>	Paired bacteriome in fat body	(b)
"	<i>Oryzaephilus surinamensis</i>	4 bacteriomes around midgut	(b)
"	Bostrichidae	Gut bacteriomes	(b)
"	<i>Lyctus linearis</i>	Fat body	(b)
"	<i>Lagria hirta</i>	Dorsal bacteriomes	(b)
"	Chrysomelidae	Midgut ceca	Nardon and Grenier, 1989; (a)
"	<i>Coccotrypes dactyliperda</i>	4 Malpighian tubules	Buchner, 1965
"	<i>Lixus</i>	Gut evaginations	(a)
"	<i>Balaninus</i>	Posterior bacteriocytes	(a)
"	Apion	2 Malpighian tubules	(a)
"	Aspidapion	Midgut	(a)
Yeasts			
Pyrenomyces	planthoppers	Fat body	Noda <i>et al.</i> , 1995
Candida	Cerambycidae	Gut mycetome	(a)
Torulopsis	Anobiidae	Gut mycetome	Bismanis, 1976
"	<i>Asteopteryx styraci</i>	Fat body	Fukatsu <i>et al.</i> , 1994
"	<i>Pimpla turionellae</i>	Various tissues	Middeldorf & Ruthmann, 1984

(a): Buchner, 1965; (b): in Schwemmler and Gasner, 1989.

4.4. INSECTS AS HOSTS

Among animals, insects are the most-studied from the symbiosis point of view. At the present time, only some families seem devoid of endocytobiontes (for instance the Carabidae) (Buchner, 1965 - Nardon and Grenier, 1989 - Nardon and Nardon, 1998). The main types of partners of insects in symbiosis are viruses, yeasts and bacteria [briefly summarized in table 1].

4.4.1. Viruses as endocytobiontes

It is not usual to consider viruses as symbiontes and there are some examples in insects where viruses can be non-pathogenic (Reisser, 1992) or useful, and heritable. Homoptera are frequently vectors of plant viruses, but they are not affected themselves. Some viruses can replicate in their vectors and can be vertically transmitted through the oocytes (Louis, 1990).

The S virus of *Drosophila/simulans* (DSV) is a causative agent of some abnormalities such as the absence of bristles in the adult (Louis *et al.*, 1988).

Some parasitoid wasps (Hymenoptera Braconidae and Ichneumonidae) like *Cotesia congregata* carry virus particles (polydnavirus) that are injected into the host (a caterpillar of *Manduca sexta* for instance) with the eggs. Viral DNA sequences become integrated in the genome of the wasp. As a consequence, the host is immunosuppressed and the parasite is not killed (Beckage, 1998). The virus particles are formed in the ovarian calyx, between the ovary and the oviduct. The nuclei of calyx cells house virions. When the cells lyse, these virions are released into the calyx lumen, where the eggs are contaminated. Even if they are not very spectacular, the viruses of insects induce ultrastructural modifications in host cells, both in the nucleus (disappearance of nucleoli, rupture of nuclear envelope), and in hypertrophied infected cells with swollen endoplasmic reticulum (formation of annulate lamellae, enlargement and vacuolization of mitochondria) (De Buron and Beckage, 1998). These effects vary according to the pathogenicity of viruses.

4.4.2. Yeasts as endocytobiontes

They are principally known in Homoptera and in Coleoptera (for more details see Houk and Griffiths, 1980 - Nardon and Grenier, 1989). In planthoppers, mycetocytes are part of the fat body and harbor yeasts of the *Candida* genus (Eya *et al.*, 1989). In scales yeast-like endocytobiontes are described (Tremblay, 1997). In *Ceroplastes rusci* their number per insect is estimated to be 60,000 – 70,000. These yeast-like microorganisms can freely float in the hemolymph of the host and occasionally in fat cells. But in *Sphaerolecanium prunastri* and *Eulecanium tiliae*, endocytobiontes are localized in large and polyploid mycetocytes, which are probably transformed fat cells.

In Coleoptera yeasts are present in numerous species of Cerambycidae (*Candida*) and in Anobiidae (*Torulopsis*) (Bismanis, 1976). There is a close resemblance between these two families. Mycetomes are associated with the intestine which forms evaginations at the beginning of the larval midgut. The number of lobes depends on species. In these intestinal protrusions not all the cells are infected [figure 5A].

Non-infected epithelial cells have a brush border while mycetocytes are giant cells without microvilli. Their cytoplasm is filled with symbiotic yeasts enclosed in vacuoles, and some of them are expelled into the gut lumen, as in the cerambycids *Rhagium inquisitor* (Ekblom, 1931) and *Criocephalus rusticus* (Riba and Chararas, 1976). Therefore yeasts exhibit two phases, intracellular and extracellular, an intermediate situation between endocytobiosis and endosymbiosis. Inside mycetocytes yeasts can be located either at the apex (Ekblom, 1931) as in *Rhagium*, or at their base as in *Leptura rubra* (Heitz, 1927). At the end of larval growth mycetomes desintegrate and some yeasts penetrate the new imaginal epithelium and induce the formation of smaller adult mycetomes. In the female symbiotes multiply in the midgut and invade the transmission devices of the ovipositor [figure 5B]. This is a strange adaptation to assume the transmission of yeasts to the progeny. When an egg is laid, the apparatus functions as a syringe and a drop containing yeasts is propelled on the egg. When the young larva emerges from the egg, it eats yeasts with the egg envelope and becomes infected. It is assumed that a long time was needed for such coadaptation occurred between these insects and yeasts.

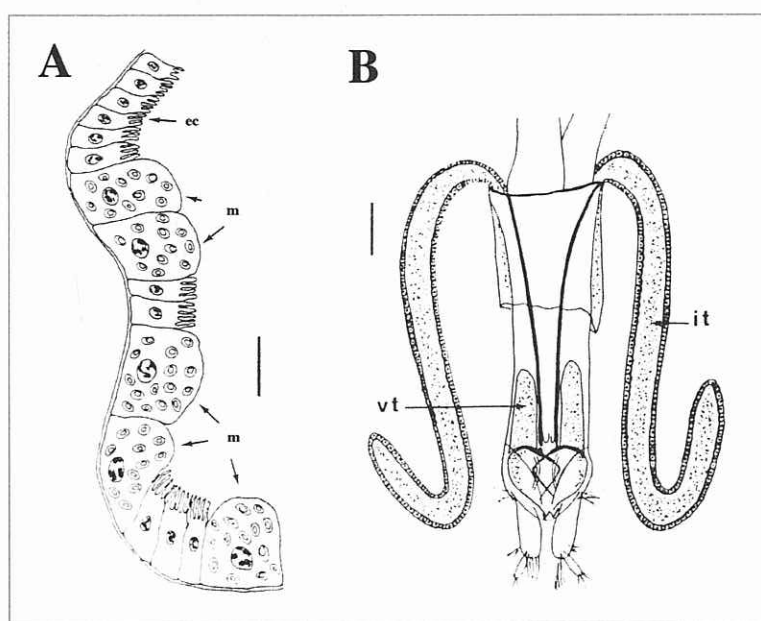


Figure 5. A: Schematic representation of the gut wall in *Stegobium paniceum*. m: mycetocyte, ec: non infected epithelial cells. Bar = 10 μ m (from Buchner, 1965, with permission of *Annals Soc. Entomol. Fr.*). B: Egg-laying apparatus of *Stegobium striatum*. it: intersegmental tubules filled with symbiotic yeasts, vt: vaginal pockets. Bar = 100 μ m (from Buchner, 1965, with permission of *Annals Soc. Entomol. Fr.*).

4.4.3. Bacteria as endocytobiontes

As shown in Figure 6, endocytobiotic bacteria of insects can be divided into five major groups: (1) the flavobacteria-bacteroid group which includes the cockroach and termite endocytobiontes; (2) the spiroplasma with only one representative (the endocytobionte of

the psyllid *Antonina crawii*); (3) the α -proteobacteria, a very homogeneous monophyletic group corresponding to the genus *Wolbachia* (Werren, 1997); (4) the β -proteobacteria (mealybug endocytobionts); (5) the highly diversified polyphyletic group of γ -proteobacteria. From another point of view, Nardon and Grenier (1993) distinguished two main types of endocytobionts. The first group is represented by the α -proteobacterium *Wolbachia* (associated symbiont), and the second is composed of integrated symbionts.

Associated bacteria: Wolbachia and others. There is now a huge bibliography concerning *Wolbachia* (see in the present book). *Wolbachia* has been found not only in numerous insects (whose number is increasing), but also in other arthropods and invertebrates (like nematodes). Symbiotic (or parasitic) status of *Wolbachia* is still controversial mainly because it is not completely "domesticated" by the host. Furthermore, the precise location in the host or in the infected cells is not clearly established. As an example, in the coleopteran *Callosobruchus sinensis*, *Wolbachia* has been detected by PCR from all tissues and organs: fat body, gut, muscle, ovary, wing, leg, head and antenna (Kondo *et al.*, 1999). In this insect, all tested individuals from 6 different populations of central Japan were infected. However, it is not always the case and very often, all the populations are not infected, and, in an infected population, all the individuals are not contaminated. This is the case in the weevil *Sitophilus oryzae* (Heddi *et al.*, 1999). Maillet (1971) studied several species of Auchenorrhyncha (Homoptera) harboring Rickettsiae (α group of *Wolbachia*). He described that the bacteria may be free in the cytoplasm or included in vacuoles. They are located in the cytoplasm or in the nucleus, and also all tissues could be infected. In *Sitophilus oryzae*, *Wolbachia*, in some strains, cohabits with the principal endocytobiont within the bacteriocytes. This phenomenon is also found in tsetse fly *Glossina*, where *Wolbachia*, in some strains, cohabits with two other endocytobionts: *Wigglesworthia* and *Sodalis* (Cheng *et al.*, 2000).

In *Glossina*, we observe different situations concerning their location: restricted to reproductive tissues in *G. brevipalpis* and *G. morsitans*, but also present in somatic tissues in *G. austeni*. The presence of *Wolbachia* in different populations of *Trichogramma* is highly variable (Pintureau *et al.*, 2000). This diversity is perhaps the consequence of an evolutive coadaptation. In infected *Trichogramma* (Hymenoptera), *Wolbachia* are concentrated at the posterior pole of the egg and are dispersed by embryonic movements. Finally they are present at a high density in the ovaries at the end of larval growth. The green rice leafhopper *Nephotettix cincticeps* harbors three types of endocytobionts: A and B types are located in bacteriomes and the bacteriocytes of ovarian pedicels, while *Rickettsia* has been found in almost all tissues, and principally in the nucleus (Mitsunashi and Kono, 1975). The bamboo Pseudococcid *Antonina crawii* also harbors three types of endocytobionts (Fukatsu and Nikoh, 2000). Two are identified as β and γ proteobacteria, and are found in the bacteriome. The third is very close to *Spiroplasma*. It is never in the bacteriome, but in various other tissues.

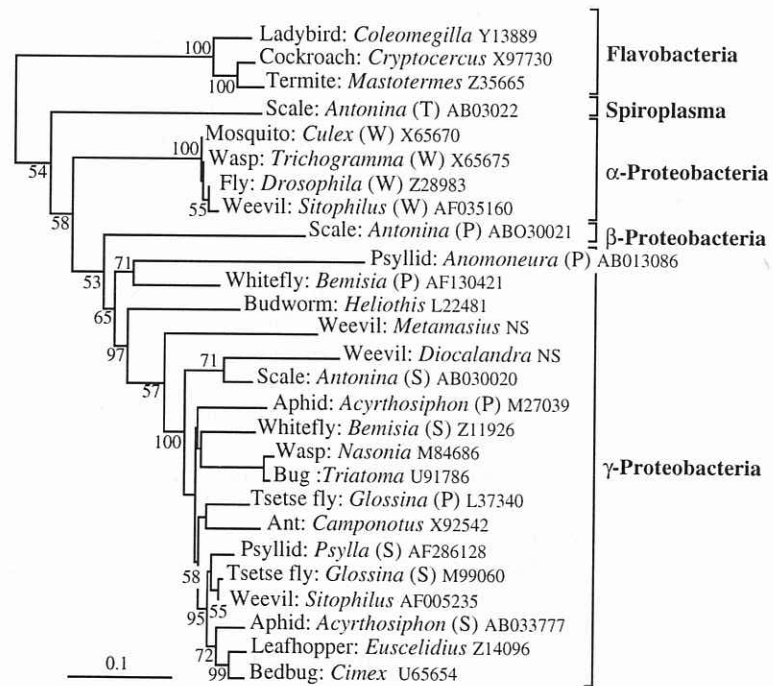


Figure 6. Neighbour-Joining phylogenetic tree of some insect endocytobionts based on 16S rDNA gene sequences and built with the distance of Galtier and Gouy (1995) accounting for unequal sequence base compositions. Endocytobionts are presented with their host common name, followed by the host genus name and the Genbank accession number. P, S, T: primary, secondary and tertiary endocytobionts. W: endocytobionts of the *Wolbachia* group. NS: non submitted sequences (Lefevre, personal communication).

To summarize, the location of these associated endocytobionts is not precise (except perhaps in eggs for some of them), they do not induce the formation of special cells or devices and they are not always in all populations. Furthermore, in an infected population, they are not always found in all individuals. This indicates that they are not obligate and their influence on the fitness of their hosts, most of the time, is negligible. That is the reason why *Wolbachia*, according to the above authors, may be considered either as a parasite or an endocytobiont.

Integrated endocytobionts: general characteristics. As seen above, most of the bacterial endocytobionts are Gram-negative. Among exceptions in insects are the symbiotic bacteria of the human body louse *Pediculus humanus* (Eberle and McLean, 1983), and those of the *Ambrosia* beetle *Xyleborus ferrugineus* (Peleg and Norris, 1972) that have been described to be Gram-positive. We have proposed the term "integrated endocytobionts" for such cytotobionts, since they are always present in the populations of a given species (in contrast to *Wolbachia*) and are completely dependent on the host for their growth. They are not cultivable *in vitro* (Nardon and Grenier 1993) and are obligate for the host. As rare exceptions, there are weevils *Sitophilus oryzae*, *S. zeamais* and *S. granarius* (Nardon, 1973 – Nardon and Grenier, 1988), in which natural and experimental populations can be devoid of symbionts. The host is greatly affected but

are able to survive and to reproduce under certain conditions (Nardon, 1973). The comparison of symbiotic and aposymbiotic insects allows the study of morphological and physiological effects of the endocytobionts. In the case of symbiote-free *Sitophilus oryzae*, the weevils have a smaller size and a lighter color.

The morphological aspect of endocytobionts varies between the species, but this is not obligatory. For instance, bacteria of the weevils *Sitophilus oryzae* and *S. granarius* are very similar, being more or less long bacilli. But we may have a diversification according to geographical strains. In a strain of *S. oryzae* from Benin, symbiotic bacteria are always long (10-30 μm) as compared with another strain from Guadeloupe that do not exceed 10-15 μm . Another example of variation is found in the whitefly *Bemisia tabaci* (Costa *et al.*, 1995). Insects from Florida, Arizona, and Hawaiï (B biotype) have two morphological types of bacteria inside bacteriocytes (coccoid C1 and P, pleomorphic), while insects of A biotype from Arizona and Mexico contain three distinct morphological types: P, C1 and C2. The authors suggested a possible coevolution between the host and its endocytobionts. The size of endocytobionts may also change according to the physiological state: in the coleopteran *Oryzaephilus surinamensis*, symbiotic bacteria are the longest in the pupa (60 - 70 μm) and appear as short bacilli (3 to 6 μm) in the ovaries of adult beetles (in Nardon and Grenier, 1989). Finally, two close species like *S. oryzae* and *S. zeamais* (sibling species) are only easily distinguished by the morphology of their endocytobionts, bacilliform in the first, and curved or spirals in the second [figure 7]. Concerning *S. zeamais* where the bacteria are so variable, the question arises: "Are these different forms the expression of a pleomorphy or the manifestation of the existence of several genetical types?" So far, in *S. zeamais*, only two types of endocytobionts have been recorded (Dash, 1975 - Campbell *et al.*, 1992), but it is not known which type corresponds to which morphology. All insect endocytobionts are not always flexuous bacilli. In aphids they are roundish, 3-6 μm in diameter, but in nurse cells of *Metamasius hemipterus* Y forms may be seen (Nardon *et al.*, 1985). It seems that these endocytobionts are larger than their free-living relatives (Enterobacteriaceae for weevils and aphids). *E. coli* is generally 0.4-0.7 μm wide and 1.0-3.0 μm long, while the *S. oryzae* bacilli are 0.3-0.5 μm wide and 4.0-30.0 μm long (Nardon, 1971).

Ultrastructural studies show that most of the symbiotic proteobacteria are typically Gram-negative. Nevertheless, particular structures can be found. For instance, in the aphid *Rhopalosiphum padi*, the endocytobionts contain paracrystalline components (Akhtar and Emben, 1994). In Homoptera various other bodies can be seen inside the endocytobionts, like the electron-dense inclusions in the cytoplasm of the "t" symbionts in *Euscelis* or *Helochora* (Hook and Griffis, 1980 - Körner, 1978).

As described by Buchner (1965), the host evidently controls the density of its endocytobionts. This has been demonstrated in the weevil *Sitophilus oryzae* (Nardon *et al.*, 1998), but the physiological mechanism is not known. One possibility is the destruction by lysosomes of supernumerary bacteria. This is sustained by lyse pictures (myelin-like structures) in bacteriocytes of insects and other invertebrates, such as *Sitophilus* (Nardon, 1971), aphids (Hinde, 1971a), or the whitefly *Bemisia tabaci* (Costa *et al.*, 1993).

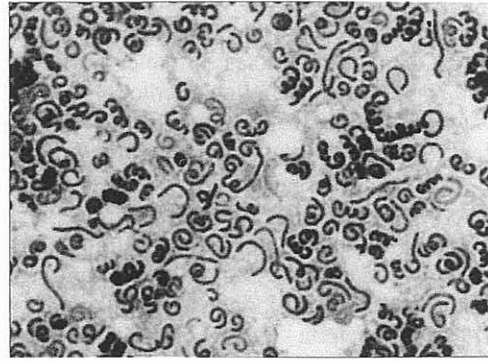


Figure 7. Endocytobionts of *Sitophilus zeamais*. Squash of bacteriome. Methylene Blue. Bar = 10 μ m (photograph by P. Nardon with permission of *Annals Soc. Entomol. Fr.*).

Location of integrated endocytobionts in bacteriocytes. In insects, endocytobionts are almost exclusively located in the cytoplasm of bacteriocytes. Nevertheless, some exceptions are known. In two termite species collected in the wild, *Kalotermes flavicollis* and *Reticulotermes lucifugus*, symbiotic bacteria were found both in the nucleus and the cytoplasm of female germ cells (Grandi *et al.*, 1997). We do not know if these observations can be extended to the whole species.

Endocytobionts are generally included in host cell vacuoles (symbiosomes). Usually only one bacterium is inside a vacuole, but, occasionally, two or more symbionts are seen in the same envelope, probably arising from recent divisions (Hinde, 1971b). How the endocytobionts penetrate into bacteriocytes is not yet known, and a phagocytic process is generally believed to occur for the formation of symbiosomes. But the reality appears more complex, and from observations of several authors (Louis, 1980 – Hinde, 1971 – Daniel and Brooks, 1972 – Chang and Musgrave, 1972, 1973 – Grinyer and Musgrave, 1966 – Louis *et al.*, 1976), we can distinguish three kinds of host/endocytobiont interface: the most widespread is the symbiosome formation. Sometimes bacteria lie free in the cytoplasm (and in nucleus) of the host cell. This is the case in the weevils *Sitophilus* (Nardon, 1971) and other Rhynchophorinae (Nardon *et al.*, 1985), and in the glossine midgut (Hübner and Davey, 1974). The “companion symbionts” in the leafhopper *Heliochara communis*, the “RC” symbionts of *Cimex lectularius* are also among endocytobionts not included inside vacuoles. Sometimes, a free endocytobiont is surrounded by a halo in fixed preparations (Nardon, 1971). This halo is not a true capsule and is interpreted as a reaction of the host cell (Louis, 1980). When an insect harbors several types of endocytobionts (plurisymbiosis), as in *Cimex lectularius*, the mode of inclusion in the cell appears symbiont-specific since in the same host cell coexist the primary endocytobiont in symbiosomes and the secondary endocytobiont which is free in the cytoplasm (Chang, 1975; Hypsa and Aksoy, 1997). But in other cases the host can influence the location of symbiotic bacteria: the endocytobiont of *Glossina* would be free in the midgut cells, but encapsulated in the

ovaries (Hübner and Davey, 1974). The "t" symbiotes of *Euscelis plebejus* are free only in bacteriocytes (Louis *et al.*, 1976), not in the embryo (Körner, 1976).

In certain insects, like pseudococcines or *Pediculus*, endocytobionts always form microcolonies inside large chambers. These "enclaves" are filled with a proteinaceous and mucous substance (Louis, 1967). Such "Mucous enclaves, as simple vacuoles, can be considered as a protection for bacteria in some circumstances, since they are destroyed when they escape in haemolymph, as in aphids (Hinde, 1971a) or in *Pseudococcus adonidum* (Louis, 1980).

It is to be noted that endocytobionts are not always intracellular. In the weevil *Sitophilus*, they are always in the germ cells, except at the beginning of embryogenesis, when they are in the egg. In other insects, where the germinal line is not infected permanently, those bacteria must leave bacteriocytes to penetrate the ovaries. During this step, endocytobionts are extracellular (for more details see reviews: Nardon, 1988; Nardon and Nardon, 1998).

Bacteriocytes, bacteriomes: structure and location. The host cells harboring bacterial endocytobionts, bacteriocytes, are either dispersed in the body, especially in adipose tissue, or in intestine, or grouped to form bacteriomes. In the carpenter ants *Camponotus* bacteriocytes are interspersed between epithelial cells of the midgut. They are filled with rod-shaped Gram-negative γ -proteobacteria, lying free in the cytoplasm. They are also present in the ovaries (Shröder *et al.*, 1996). As for all insect endocytobioses, bacteria have no influence on the ovocyte morphology. On the contrary, bacteriocytes always appear as specialized and modified cells. Concerning the ant bacteriocytes they differ from normal epithelial cells by the absence of microvilli at their apical part. In cockroaches, bacteriocytes are disseminated among the fat body. They are relatively large, 256-512 ploid cells (Richards and Brooks, 1958; Sacchi and Grigolo, 1989). All aphids have an obligatory association with microorganisms, with the exception of phylloxerines. The endocytobionts are generally bacteria. Yeasts have only been found in some cerataphidini, like *Astegopteryx styraci* (Fukatsu and Ishikawa, 1992; Fukatsu *et al.*, 1994). The bacterial endocytobionts are always within vacuoles. The bacteriocytes, singly or in small aggregates, are found beneath the intestine, within the abdomen of adult aphid, and around the gut in developing embryos. These bacteriocytes are huge cells, reaching 40-60 μm in diameter, and polyploid. They possess all cell organelles, and also vesicles and granular bodies. Microtubules and microfilaments have also been identified (Akhtar and Van Emden, 1994) in *Rhopalosiphum padi*. The cytoplasm is very rich in ribosomes and also contains a great number of vesicles which lie close to the symbiosome membranes (Houk and Griffiths, 1980). In the pea aphid *Acyrtosiphon pisum* and in some other species, two different endocytobionts are present (Unterman *et al.*, 1989). The most important is the cocciform primary symbiote (P), located inside typical bacteriocytes. The secondary symbiote (S) is smaller and bacilliform. It is located in cells which generally surround the bacteriocytes (Fukatsu *et al.*, 1998).

Many other insects are plurisymbiotic, permanently or occasionally. Furthermore the bacteriocytes, most of the time, are compacted to form symbiotic organs: the bacteriomes. The location of these bacteriomes seems to be under the control of the insect, since they are generally found in the same place for different species of a same genus or family, or sub-family. For instance, all the Rhynchohorinae examined so far, except *Sitophilus linearis* which is asymbiotic, possess a compact larval bacteriome at

the junction foregut/midgut (Nardon *et al.*, 1985; Nardon and Nardon, 1998). During metamorphosis, bacteriocytes migrate and invade the anterior mesenteric caeca of the adult, where they stay for about three weeks. Afterwards they disappear, so that in weevil more than three weeks old, the only symbiotes in the female are those contained in oocytes, trophocytes and apical bacteriomes of the ovaries (Nardon, 1971; Nardon and Grenier, 1988). Endocytobiosis often differs between larvae and adults. The bacteriome must be considered as a new organelle, dependent on the physiology of the host, as other organelles, also remodulated during metamorphosis.

The bacteriomes are single as in *Sitophilus*, or paired. As in numerous species with one or two compact larval bacteriomes, the *Sitophilus* bacteriome is limited by a sheath of small flattened cells, and is composed of polyploid bacteriocytes, rich in ribosomes, and of small accessory cells. It possesses numerous trachae. In *Rhizopertha dominica* and other Bostrichidae, the structure of paired bacteriomes is a little different since the center is occupied by a multinucleated syncytium. In *Lyctus linearis*, paired bacteriomes lie close to the lateral lobes of the fat body, between midgut and gonads. It is formed of a total of 7 - 12 syncytia (50 to 100 μm) with irregular nuclei centrally arranged, and surrounded by 8 - 14 smaller ones with round nuclei. The syncytia are embedded in epithelial cells, and each type contains one endocytobiont (Gambetta, 1927). It would be interesting to verify this point with modern techniques.

In Homoptera, numerous types of plurisymbiosis are described. In psyllids the bacteriomes, deeply penetrated by tracheoles, lie between gonads and the alimentary canal. It is 500 to 700 μm long in *Psylla pyricola* (Chang and Musgrave, 1969). It is composed of a central syncytium, of peripheral bacteriocytes and of a cellular covering sheath. Two types of endocytobionts have been described in *Anomoneura mori*: X and Y (Waku and Endo, 1987), now identified as γ -proteobacteria, but belonging to different lineages (Fukatsu and Nikoh, 1998). The primary (P) symbiotes are in the bacteriocytes, while the secondary (S) symbiotes are in the syncytial region of the bacteriome (Spaulding and Von Dahlen, 1998; Thao *et al.*, 2000).

It is not possible, here, to present all aspects of endocytobiosis. Some insects, like *Laodelphax striatellus*, harbor both bacteria and yeasts (Noda *et al.*, 1979). According to Buchner (1965), 55% of the cicadas would be disymbiotic, 30.5% trisymbiotic, 4.2% tetrasymbiotic, 1.5% pentasymbiotic, 0.5% hexasymbiotic, with only 5.4% monosymbiotic and 2.5% without symbiote. Concerning the location of bacteriomes five main types may be distinguished: gut caeca, midgut, compact organs associated with the midgut, fat body and malpighian tubules.

5. Discussion and conclusion

De Bary (1879), in his definition of symbiosis, was already convinced that all degrees of intimacy existed between associated partners, and notably between mutualism and parasitism (see in Nardon and Grenier, 1993). It is sometimes difficult to trace a frontier, and we have seen that the bacterium like *Wolbachia*, may be considered either as a parasite or a symbiote, according to the host species and the author. One remarkable feature is that *Wolbachia* seems to have no morphological influence on the host cell. In his book, Combes (1995) proposed to replace the De Bary concept of symbiosis by the new concept of "durable interactions", in order to avoid some

confusion about the quality (useful or not) of the association. This is certainly a good idea from a genetic point of view, but difficult to assume physiologically. Symbiosis is not simply an interaction, but also an innovative mechanism (Margulis and Fester, 1991). In fact, it appears that this new concept is essentially adopted by the parasitologists. Why? Probably because the neoDarwinism puts forward the notion of conflict, more familiar to parasitologists than to symbiologists, whose approach is quite different, with the notion of complementarity between the partners. Even if we can observe a continuum between parasitism and mutualism, it is nonetheless evident that the two types of associations can be easily distinguished. The example of bacteria infecting *Amoeba proteus* is demonstrative since the same endocytobiont pathogenic in a first step, became symbiotic after only 200 generations, and since the two situations were perfectly distinguishable. We have proposed (Nardon and Grenier, 1993) the concept of "symbiocosm" which represents the new biological entity created by symbiosis, and submitted to natural selection. The notion of symbiocosm supposes that different genomes interacting (nuclear, mitochondrial and / or plastidial, symbiotic) have reached an equilibrium. During the phase of adaptation, in some associations, the morphology of the partners are not, or seldom modified (contrarily to physiological ways): an example is the association *Hydra* / *Chlorella*. But, generally, in all types of symbiosis, almost all partners in the symbiocosm, have been modified for some morphological features. For instance, bacteriocytes and mycetocytes are hypertrophied and polyploid cells in all cases. In the squid, appendages disappear when symbiosis begins and the *Vibrio* loses its flagella. In *Rhizobium* legume symbiosis, the two partners are modified, and exchanges of signals are almost completely discovered. In this example it is the bacterium that directly induces the nodule formation. In *Sitophilus oryzae*, the larval bacteriome and apical bacteriomes of adult female ovaries disappear in aposymbiotic (=deprived of symbionts) insects. This suggests that a signal from the symbiotic bacteria trigger the differentiation of bacteriomes. But we do not know if such a situation is general. It does not seem, since in *Oryzaephilus surinamensis* bacteriomes are also formed in the absence of bacteria through 25 generations (Koch, 1967).

The modification of the partner morphology can be spectacular and lead to the formation of complex structures, or symbiotic organs: galls, trophosome, luminous organs, lichens, mycangia... Some of these mycangia disappear in the absence of symbionts. But such an inducing power is not always easy to verify. It is possible that in certain symbiosis, and especially in ectosymbiosis, some structures would be more ancient than the association and secondarily exploited. That could be the case in the ant/plant symbiosis, where domatia are not induced by ants. Rahat (1990) also evokes the concept of preadaptation to explain the association of algae with *Hydra*. It appears to us that the phenomenon of symbiosis is more frequent in invertebrates than in vertebrates. It allows surviving in various conditions, by complementarity. On the contrary, in the more complex vertebrates the parasitism appears more frequent than symbiosis, perhaps in relation to a better system of defence. These morphological structures of symbiosis are a good illustration of what R. Dawkins (1982) termed as the "extended phenotype", that is the possibility for a gene to be expressed outside the organism. These genes which control the symbiotic phenotype are necessary for the maintenance of the symbiocosm (symbiotic gene). They also create new structures. But we have tried to show that innovations and transformations have common

characteristics in all types of symbiosis. The biodiversity of symbiosis mainly corresponds to the biodiversity of the partners.

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