

Review article

Microbial Symbiosis in Annelida

MONIKA BRIGHT^{1*} and OLAV GIERE²

¹*Institute of Ecology and Conservation Biology, University of Vienna, Althanstrasse 14, A-1090 Vienna, Austria, Tel. +43-14277-54331, Fax. +43-14277-54339, Email. monika.bright@univie.ac.at;*

²*Zoologisches Institut und Zoologisches Museum, Universität Hamburg, Martin-Luther-King-Platz 3, D-20149 Hamburg, Germany, Tel. +49-42838-3865, Fax. +49-42838-3937, Email. olav.giere@zoologie.uni-hamburg.de*

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Abstract

Among Annelida various taxa have independently established symbioses with numerous phylotypes of bacteria, the most renowned symbiosis being that of the giant tube worms around hydrothermal vents. Taxonomically, the symbiotic associations range on the host side from a few genera within else aposymbiotic groups (Tubificidae in the Oligochaeta or Glossiphoniidae in the Hirudinea) to higher taxa with all their species living in symbiosis (Siboglinidae, Alvinellidae in the "Polychaeta"). The ties between partners range from rather loose and occasional associations (some mud-dwelling tubificids) over regular ectosymbiosis (Alvinellids) to obligatory extra- or intracellular incorporation into the body (gutless oligochaetes, glossiphoniid leeches, siboglinid tube worms). Occurring from eulittoral beaches to deep-sea hot vents, from rotting wood at the sea bottom to freshwater ponds, the associations with bacteria seem to have adaptive significance in a broad ecological and nutritional range. In the sea, chemoautotrophy in sulfidic, otherwise hostile environments is a major factor driving the symbioses, but trophic specializations such as digestion of wood or blood also seem to favor a coordinated and mutually beneficial association with bacteria.

*The author to whom correspondence should be sent.

While most annelid hosts harbor just one bacterial phylotype, the symbioses in the rich species flock of bacteria-symbiotic gutless oligochaetes (*Olavius* and *Inanidrilus*) are dual or even multiple. Most sulfide oxidizing bacterial symbionts belong to the gamma-Proteobacteria, some delta-Proteobacteria have been found to reduce sulfate. The alpha-Proteobacteria, isolated from various hosts, are suggested to participate in nitrogen metabolism. The function of symbiotic Spirochaeta is unknown. Principal impairments in studies on annelid symbioses are the impossibility to experiment with bacteria-free hosts and to re-infect them with the symbiotic bacteria cultured *in vitro*. Description of the various symbioses follows the taxonomic grouping of the hosts; structural, microbiological and functional aspects of the symbioses are considered.

Keywords: Symbiosis, thiotrophic, methanotrophic, nephridia, Siboglinidae, *Olavius*, *Inanidrilus*, *Alvinella*, bacterial phylotypes

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1. Introduction

A variety of bacteria-symbioses are known in annelids, but only a few of them have been studied in detail. Certainly, they came to our attention through the discovery and research on hydrothermal vents, but the bacteria symbioses in non-vent meiobenthic annelids from shallow sediments (Oligochaeta) were described simultaneously. We will concentrate on the symbiosis of annelids with one or several bacterial phylotypes regardless of their extra- or intracellular position, their nature as ecto- or endosymbionts, and the type of tissue involved. The symbiosis of the Siboglinidae, recently reviewed by McMullin et al. (2003), will be supplemented with current literature on morphology and ultrastructure and otherwise considered in brief. The symbiotic association of intracuticular bacteria with *Questa paucibranchiata* (Polychaeta, Questidae) (Giere, 1996; Giere and Erséus, 1998) and of intraepidermal bacteria in nerillid polychaetes (Tzetlin and Saphonov, 1995) will be disregarded since details on these associations are lacking.

General aspects of taxonomic affiliation and of relevant environmental factors will precede comments on the structural and microbiological details of the symbiosis. Largely based on molecular analyses, considerations on functional aspects will conclude each chapter.

2. Symbiosis in Chemosynthetic Environments

In environments where energy production is based on chemosynthesis, symbioses between annelids and bacteria are a regular phenomenon. These associations are extremely diverse regarding anatomical, physiological, and biochemical aspects. They differ in the wide range of annelid hosts and in the benefits that the different hosts gain from their specific symbionts. Such symbioses have evolved several times in a variety of annelid groups indicating their significant evolutionary role.

Symbioses have been reported from many habitats where sulfide is produced either geothermally (vents) or biologically (seeps, sediments): hydrothermal vents, hydrocarbon cold seeps, or reducing shelf sediments. These habitats are generally characterized by temporal and/or spatial fluctuations of oxygen and hydrogen sulfide (Fisher, 1996). Life under these unstable, fluctuating physico-chemical conditions challenges the host facing temporal anoxia combined with considerable amounts of one of the most toxic chemicals – hydrogen sulfide (National Research Council, 1979).

Chemoautotrophic thiotrophic bacteria oxidize reduced sulfur species such as hydrogen sulfide or thiosulfate and use the energy gained for inorganic carbon fixation. They are found as intracellular endosymbionts in the majority

of Siboglinidae, as extracellular endosymbionts in the oligochaete genera *Olavius* and *Inanidrilus* (Tubificidae), as well as ectosymbionts in representatives of the polychaete genus *Alvinella* (Alvinellidae). *Alvinella* and some of the oligochaetes possess, in addition to their primary thiotrophic symbionts, one or several other phylotypes mostly of unknown function. The only example of methanotrophic bacteria utilizing methane as a sole source of carbon and energy is found in the frenulate pogonophore *Siboglinum poseidoni*. Several additional microbial associations are reported from chemosynthetic environments, and thiotrophic physiology has been suggested for the associated microbes.

Endosymbiosis in Siboglinidae (Polychaeta)

Taxonomical remarks

Considerable literature has been published on a variety of symbiotic aspects in the former phylum Pogonophora (Caullery, 1914). In the course of time, Pogonophora changed its taxonomic affiliation, rank and subdivision several times, so that a definition and clarification seems adequate here. With the discovery of the hydrothermal tube worms, a taxon Vestimentifera (or Obturata) was established either as a new subtaxon of Pogonophora ranked in parallel to the earlier known subtaxon Frenulata (or Perviata), or as a new phylum outside the earlier phylum Pogonophora. Today, the Perviata contain the earliest known genus *Siboglinum*, plus, among others, the genera *Nereilinum*, *Spirobrachia*, *Lamellisabella*, and – in some taxonomic distance – *Sclerolinum*.

The Vestimentifera or Obturata, on the other hand, are renowned through the huge tube worms *Riftia* ornamenting in spectacular thickets the deep-sea hydrothermal vents. Also, the less known genera *Lamellibrachia*, *Escarpia*, *Ridgeia* and *Arcovestia* belong to this taxon. Relations of *Sclerolinum* to the Vestimentifera have been recognized (Southward, 1993) and in some classifications, *Sclerolinum* has been removed from the Perviata s.str. and classified as a sister taxon "Atheceanephria". Rouse and Fauchald (1997) consider *Sclerolinum* a sister taxon to all the other Vestimentifera (or Obturata), this combination being termed Monilifera. Monilifera then represent the pendant to the Frenulata or Perviata. Today, about 150 species of tube worms are known.

Morphological scrutiny of larval and complete adult forms (Southward, 1988; Rouse, 2001) and molecular analyses (Halanych et al., 2001) reveal with increasing probability that all these "tube worms", in fact, represent annelids. McHugh (1997) and Rouse and Fauchald (1997) consider them a derived polychaete family related the Sabellida that should, for priority reasons,

receive the name Siboglinidae. Accordingly, we follow Rouse (2001) in his connotation of Siboglinidae containing the sister taxa Frenulata and Monilifera (Vestimentifera + *Sclerolinum*).

The autapomorphy of the Siboglinidae is an occluded gut. Coinciding with the vestigialization of the alimentary tube, all the representatives live in an obligatory association with endosymbiotic bacteria. This bacterial association was discovered in conjunction with the studies on the conspicuous vestimentiferan giant tube worm *Riftia pachyptila* (Jones, 1981a,b; Cavanaugh et al., 1981; Felbeck, 1981; Rau, 1981). Since then numerous studies have dealt with various aspects of the *R. pachyptila* symbiosis, making it to the best studied of thiotrophic associations. A considerable body of literature covering a variety of symbiotic aspects also has been published on other vestimentiferan tube worms. Stimulated by the intense studies on Vestimentifera also in the less studied Frenulata, although known for a long time, a corresponding symbiosis with thiotrophic bacteria was revealed (Southward et al., 1981).

The environment

Frenulata occur worldwide in a variety of marine habitats: reducing sediments in shallow depths (20 m), fine muds of fjords, on the continental slope, in the trenches of the deep-sea (9,050 m), but also in cold seeps and on whale falls (Flügel and Langhof, 1983; Ivanov, 1963; Southward, 1971, 1979). They live at ambient temperatures with the posterior end buried in the anoxic, sulfidic sediment layer (Southward, 1982; Southward et al., 1986). So far, only one species, *Siphonobranchia lauensis*, has been discovered living in sediment-covered cracks and crevices in the vicinity of hydrothermal vents of the back arc Lau Basin (Southwest Pacific) (Southward, 1991). Some species of *Sclerolinum* such as *S. sibogae* or *S. magdalenae* live buried in mud (Southward, 1972). Other species are found in rotting wood (Webb, 1964a,b; Southward, 1972; Ivanov and Selivanova, 1992). The blackend appearance has been taken as indication that sulfide is present in this habitat.

In contrast, Vestimentifera are found on hard substrates (basalt or sulfide chimneys) around deep-sea hydrothermal vents as well as in sedimented venting areas and cold seep sediments. Vestimentifera are described from the Pacific and the Atlantic Ocean and the Mediterranean Sea (see Gardiner and Hourdez, 2003), but, as yet, there are no records from the Indian Ocean (see Van Dover et al., 2001). Mostly, they are found in the deep-sea; however, dense aggregations of *Lamellibranchia satsuma* thrive in the euphotic zone as shallow as 82 meters in Kagoshima Bay (Hashimoto et al., 1993; Miura et al., 1997). Most species seem restricted to one type of habitat and inhabit either hydrothermal vents or cold seeps. Only *Escarpia spicata*, *Lamellibranchia barhami*, *L. satsuma* and an undescribed species occur both at warm venting and cold seep environments (Jones, 1985; Black et al., 1997; Southward and Galkin,

1997; Tunnicliffe et al., 1998; Kojima et al., 2001). *Escarpia spicata* is exceptional among vestimentiferans being found on whale falls (Black et al., 1997).

Vent vestimentiferans live in an extreme unstable environment characterized by catastrophic volcanic eruptions, tectonic disturbances, and temporarily and spatially fluctuating hydrothermal vent fluids that form a transient environment (see Van Dover, 2000). In addition, vent fluid composition, flux rates, and mixing with ambient seawater is variable in dramatic and unpredictable ways (see Childress and Fisher, 1992; Fornari et al., 1998). In contrast to earlier *in situ* measurements, newly developed underwater techniques showed that the tube worms thrived under moderate sulfide conditions only with maxima of 12 μM and 31 μM respectively at Genesis 13°N East Pacific Rise (Sarradin et al., 1998; Le Bris et al., 2003) or approximately 30 μM at 9°N East Pacific Rise (Luther et al., 2001).

Ridgeia piscesae lives in a variety of flow regimes at the Northeast Pacific (see Tunnicliffe and Juniper, 1990; Southward et al., 1995; Sarrazin and Juniper, 1999). This species exhibits different growth forms and a greater tolerance to varying physico-chemical conditions than any other known Vestimentifera. This plasticity appears to be correlated with the thermal characteristics of its microenvironment and led to the distinction of the two morphotypes "short fat" and "long skinny" (Scott et al., 1999; Urcuyo et al., 1998) which earlier had also been considered two separate species.

Vestimentifera from seep environments live under more moderate conditions than their vent relatives as evidenced by the lack of temperature anomalies. Studies in the Gulf of Mexico showed that at seep tube worm aggregations hydrogen sulfide is not detectable at the plume level but is present at the posterior end buried in the sediment (Julian et al., 1999; Scott and Fisher, 1995).

Morphology and ultrastructure of the symbiosis

Evidence, so far, points to a biphasic life cycle with a non-symbiotic larva and a benthic, sessile, symbiotic adult. Uptake of symbionts seems to occur in early juveniles, after metamorphosis is completed. Subsequently the worm transforms into a 'symbiotic entity'. The most important morphological changes herein are the development of a symbiont-containing organ, the trophosome (van der Land and Nørrevang, 1975, 1977), and the parallel vestigialization of the digestive system. Their detailed description was based on light microscopical studies on the vestimentiferan *Lamellibrachia luymesii*. Although they did not identify the bacteria, they distinguished different types of pigmented granules and noticed their positive DNA staining. The first ultrastructural description recognizing the microbial symbionts of *Riftia pachytila* was done by Cavanaugh et al. (1981). Many detailed studies followed: *R. pachytila* (Figs. 1a–e; Bosch and Grassé, 1984a,b; Hand, 1987;

Gardiner and Jones, 1993; Bright and Sorgo, 2003), *Ridgeia piscesae* (Jones, 1985; redescription Southward et al., 1995; de Burgh, 1986; de Burgh et al., 1989; Malakhov et al., 1996; Malakhov and Galkin, 1998), several species of *Lamelibranchia* (Kim and Ohtu, 2000). Southward et al. (1981) and Southward (1982) investigated several species of *Siboglinum* (Fig. 1g-i) and *Sclerolinum* and introduced the name trophosome also for a corresponding structure in all Siboglinidae. Kim and Ohtu (2000) studied the trophosomal ultrastructure of two not further identified frenulates.

The trophosome lies in the coelomic cavity of the trunk region (Figs. 1a, g). Ivanov (1963) first interpreted the trophosome of the frenulates *Polybranchia annulata*, *Lamellisabella zachi*, and *Siboglinum cauleryi* as a "median coelomic sac". In *Riftia pachyptila*, the trophosome takes up about 16% of the wet weight of an adult worm (Childress et al., 1984) and extends throughout the entire trunk region reaching into the posterior part of the vestimentum. In *Riftia pachyptila* only one fourth of the trophosome's volume harbors the endosymbionts while the remaining tissue is of host origin (sheath cells, myoepithelial cells, non-bacteriocytes, and host portion of bacteriocytes) (Powell and Somero, 1986; Bright and Sorgo, 2003). In Frenulata and *Sclerolinum*, the trophosome is fairly small and restricted to the posterior portion of the trunk. It has been estimated that the bacteriocytes take up less than 5% of the total volume of the animal. Symbionts account for less than 1% of the total volume (Southward, 1982).

Both Vestimentifera and Frenulata/*Sclerolinum* exhibit a characteristic, but morphologically different trophosome (Fig. 2). Symbiont containing host-cells, termed bacteriocytes, are distinguished from cells devoid of symbionts (termed non-symbiotic cells). In Vestimentifera, the majority of bacteriocytes are part of an 'apolar' tissue (i.e. cells without apical belt-shaped junctional complexes, being submerged in the extracellular matrix; Rieger, 1994) while, in Frenulata and *Sclerolinum*, they are arranged in an epithelial structure (Fig. 2), a simple two-layered trophosome. This is composed of an inner bacteriocyctic epithelium and an outer myoepithelium. The blood vessel in between is lined by the basal matrices of both epithelia (Southward, 1982). In Vestimentifera, the trophosome is a complex, multi-layered organ composed of numerous interconnecting lobules (Figs. 1b-d, 2, 3). Each lobule contains an inner axial blood vessel with a surrounding myoepithelium, followed by a massive apolar tissue of bacteriocytes, and an outer peritoneum that covers the peripheral bacteriocytes and lines the body coelom. Interconnected "sheath cells" of the peritoneum form a network of ramifying peripheral blood vessels; these are surrounded by non-muscular epithelial cells.

A few bacteriocytes are also part of the myoepithelium surrounding the axial blood vessel in vestimentiferans. The vestimentiferan myoepithelium is composed of myoepithelial cells, non-symbiotic, non-muscular "non-

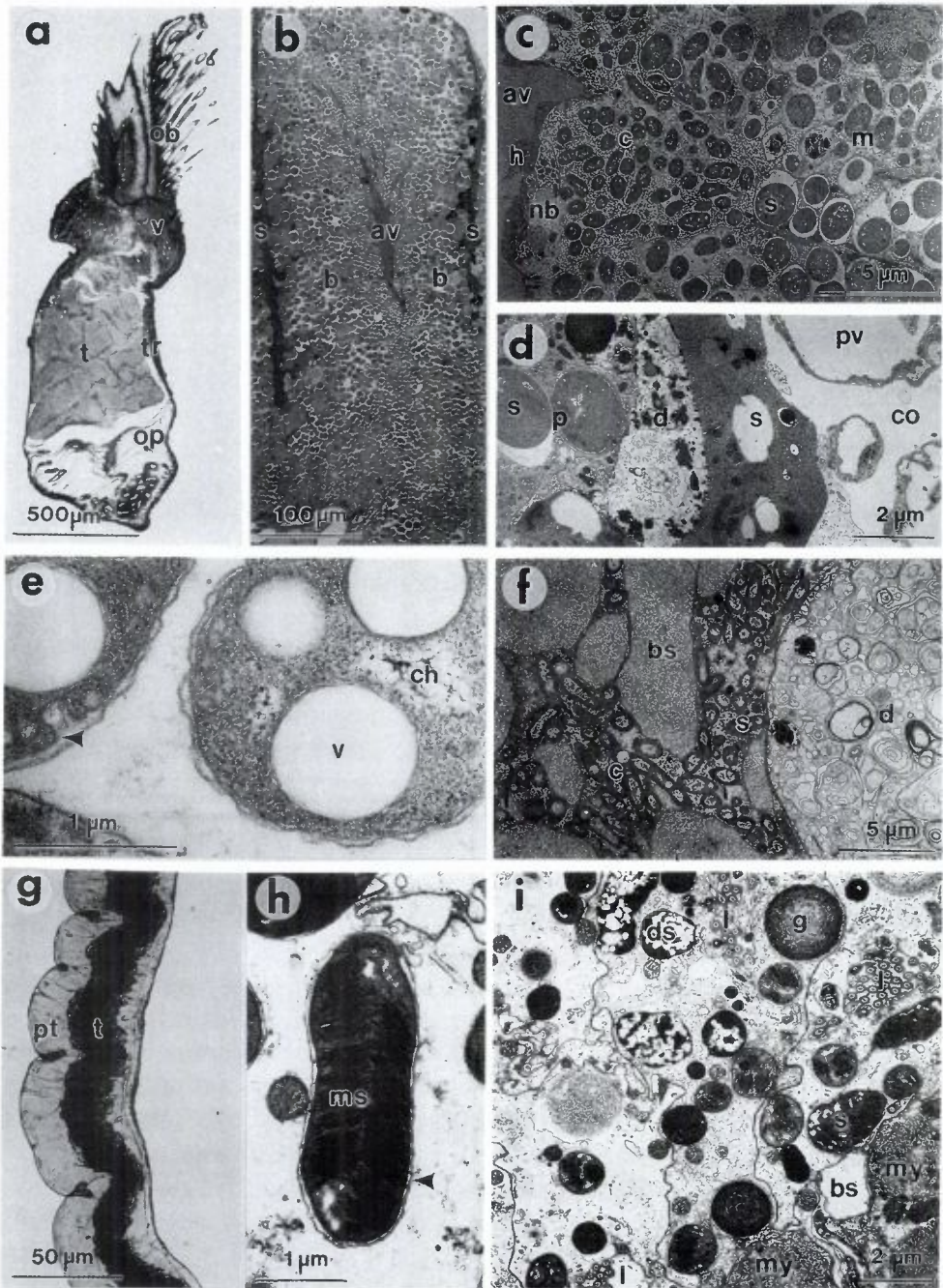


Figure 1. See legend on next page.

bacteriocytes", and a few bacteriocytes. Being centrally located, its basal matrix lines the axial blood vessel. By contrast, the myoepithelium in frenulates and *Sclerolinum* is a peripheral structure and, thus, is of opposite orientation in relation to the trunk coelom: In *Siboglinum fiordicum*, a central lumen is surrounded by the bacteriocyte epithelium. In *S. ekmani* and *S. poseidoni* (Fig. 1i) the central lumen is restricted to small spaces. In these two species cilia of the bacteriocytes extend into the remnants of the lumen. In *S. angustum* and *Sclerolinum brattstromi* the lumen is occluded (Southward, 1982; Callsen-Cencic and Flügel, 1995).

The bacteriocytes of frenulates and *Sclerolinum* appear uniform (but see Kim and Ohtu, 2000). In contrast, they are quite varied in vestimentiferans and lead to the distinction of four zones: central, median, peripheral, and degenerating zone (Bosch and Grassé, 1984a,b; Bright and Sorgo, 2003). An increase of size from the center to the periphery of each lobule results in an increase of symbiont number and size (Figs. 1c,d; Bright and Sorgo, 2003). As the cytoplasmic space between symbionts becomes smaller towards the periphery, nuclei become more and more distorted (deBurgh, 1989; Bosch and Grassé, 1984a,b). These differences in bacteriocytes and symbionts (Fig. 3) were interpreted as the result of a specific cell cycle with terminal differentiation (Bosch and Grassé, 1984a,b; Bright et al., 2000; Bright and Sorgo, 2003).

Figure 1. See figure on previous page.

Light (LM) and transmission electron (TEM) micrographs of Siboglinidae *Riftia pachyptila* (a-e), *Ridgeia piscesae* (f), *Siboglinum poseidoni* (g-i). (a). Longitudinal semithin LM section through juvenile. Ob = obturacular region; ve = vestimentum; tr = trunk with t = trophosome; op = opisthosome. Light micrograph. (b). Cross section of trophosome lobule. Av = central axial blood vessel; b = bacteriocyte area; s = sheath cells of peripheral peritoneum. Light micrograph. (c). TEM of central zone of trophosome lobule. Av = axial blood vessel containing h = hemocytes; myoepithelium, in this section with n = non-bacteriocytes and c = central bacteriocytes; central and m = median bacteriocyte zone containing s = symbionts. (d). TEM of peripheral zone of trophosome lobule. P = peripheral and d = degenerating bacteriocyte zone; s = sheath cells of peritoneum; pv = peripheral blood vessels in c = coelom. (e). TEM of thiotrophic symbionts. V = sulfur vesicles; infolding of cytoplasmic membrane (arrowhead), and ch = chromatin strands. (f). TEM of degrading trophosome. C = central bacteriocytes; bs = blood space; d = degenerative bacteriocytes. (g). Whole mount. Pt = postannular region; t = trophosome. Light micrograph. (h). TEM of trophosome. Ms = methanotrophic symbiont; symbiosome membrane (arrowhead). (i). TEM of cross section of trophosome. L = central lumen filled with cilia; s = healthy and ds = degenerating symbionts; g = granules; bs = blood space; my = myoepithelium.

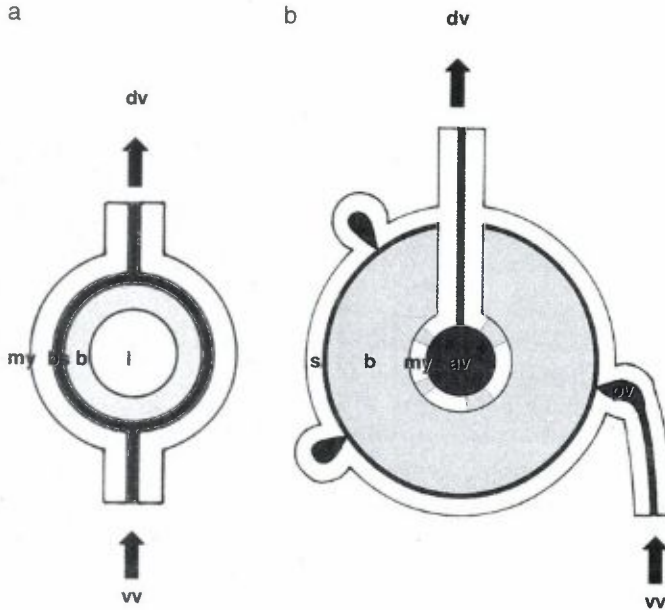


Figure 2. Schematic drawing of different types of trophosomes in Siboglinidae: Frenulates and *Sclerolinum* (a) and Vestimentifera (b). (a). b = bacteriocyte epithelium; my = myoepithelium, l = central lumen; bs = blood space. (b). av = axial blood vessel surrounded by a myoepithelium; b = bacteriocytes; s = sheath cells of peritoneum; vv = ventral blood vessel; pv = peripheral blood vessels; dv = dorsal blood vessel; note the opposite direction of epithelia surrounding the central lumen in both trophosomes (modified from Bright and Sorgo, 2003).

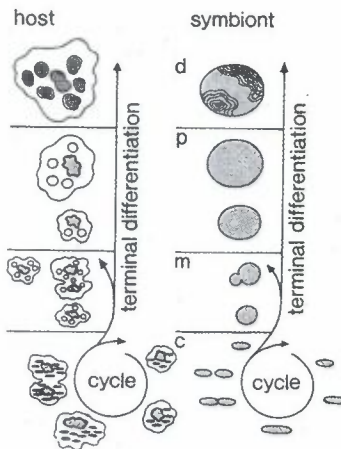


Figure 3. Schematic drawing of host bacteriocyte (left) and symbiont (right) cell cycle with terminal differentiation in *Riftia pachyptila*. c = central, m = median, p = peripheral, and d = degenerating bacteriocyte zones (modified from Bright and Sorgo, 2003).

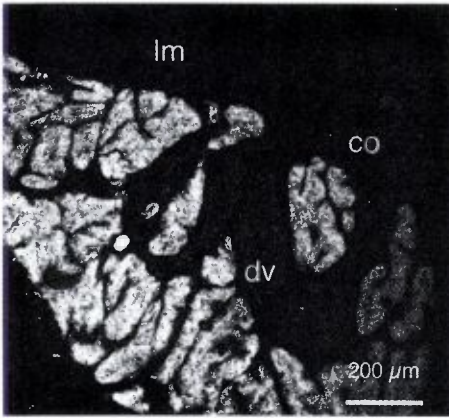


Figure 4. Micrograph of fluorescence *in situ* hybridization using a Cy3 labeled oligonucleotide probe specific for the symbionts of *Riftia pachyptila*. t = trophosome labeled; dv = dorsal blood vessel and lm = longitudinal muscles non-labeled showing weak autofluorescence; co = trunk coelom.

It has been suggested that the centrally located bacteriocytes function as stem cells proliferating towards the periphery where they become degraded. Bacteriocyte division has never been observed in any trophosome. According to the proposed cell cycle in Vestimentifera, division should be restricted to the centrally located cells whereas, in Frenulata and *Sclerolinum*, a random renewal of the bacteriocyte epithelium should occur in the entire bacteriocyte epithelium.

A conspicuous feature, only found in vestimentiferans, is the simultaneous presence of two bacterial morphotypes, rods and cocci. The vast majority of *Riftia pachyptila* symbionts are coccoid and are found in the median, peripheral, and degenerating bacteriocyte zones, which together make up 97.7% of the total bacteriocyte tissue. The remaining 2.3% of bacteriocytes in the central zone contain rod-shaped morphotypes. Excluding the degrading symbionts in the peripheral zone, 97.9% of the symbionts are cocci and only 2.1% are rods. In all vestimentiferans studied, the symbionts are mostly housed individually in membrane-bound vacuoles (termed "symbiosomes"; Bright and Sorgo, 2003).

In Frenulata and *Sclerolinum*, polymorphism of symbionts is absent. Only rod-shaped bacteria, that might be curved or S-shaped, are found in all of the species studied. *Sclerolinum brattstromi* and *Siboglinum poseidoni* symbionts

contain rather thick rods (Southward, 1982; Smaljohann and Flügel, 1987). In the Frenulata, the bacterial symbionts are enclosed in symbiosomes; in contrast, the symbionts of *S. brattstromi* appear to lie free in the host cytoplasm (Southward, 1982).

Conspicuous periplasmic sulfur vesicles (electron-transparent areas *sensu* Cavanaugh et al., 1981; particles of elemental sulfur *sensu* Southward et al., 1981, electron-lucent vacuoles *sensu* Southward, 1982) are found in all vestimentiferan symbionts and were noticed also, albeit less frequently, in frenulate symbionts. This is indicative of a thiotrophic metabolism with oxidation of reduced sulfur species (Jannasch, 1984). The membranes of these vesicles represent infoldings of the cytoplasmic membrane and the enclosed spaces are contiguous with the periplasmic space. They appear empty after standard fixation and embedding procedures for transmission electron microscopy because elemental sulfur readily dissolves in solvents such as ethanol or acetone. Proof of elemental sulfur confined to these vesicles has been provided recently in *R. pachyptila* (Pflugfelder, 2003; but see Truchet et al., 1998). Generally, the number of sulfur vesicles appears to be lower in frenulates than in vestimentiferans. In specimens of *R. pachyptila* with low overall content of elemental sulfur, sulfur density was found to be similar in both bacterial morphotypes, but in specimens with high overall sulfur content, the cocci contained significantly more sulfur than the rods (Pflugfelder, 2003). A similar relation has been revealed for glycogen density (Sorgo et al., 2002).

All thiotrophic symbionts of Siboglinidae, studied so far, share similar ultrastructural features: the cytoplasmic and outer membrane, typical for gram-negative bacteria, are usually parallel depending on quality of fixation (Fig. 1e). In rods and small cocci the chromatin strands are found in a central electron-translucent core. In the large cocci, the extension of chromatin strands appears more elaborate and the number of nucleoids has to be ascertained. The cytoplasm is electron-dense and contains ribosomes, glycogen, and sulfur vesicles.

In the Vestimentifera, the bacterial cytoplasm contains stacks of double membranes parallel to the cell membrane ("intracytoplasmic membranes" *sensu* Cavanaugh et al., 1981; "concentric membrane whorls" *sensu* Hand, 1987; "internal membrane system", "lamellar membranes" *sensu* deBurgh et al., 1989). Peripherally, they form small vesicles of a similar electron-density as the cytoplasm. The function of these membrane stacks is as yet unknown.

Granules of medium electron density, assumed to contain the bacterial storage compound poly- β -hydroxybutyrate (PHB), were noticed in the symbionts of *Ridgeia piscesae* (deBurgh et al., 1989) but have not been detected in *R. pachyptila* (own unpubl. obs. M.B.) or in any other symbionts. Although structures similar to carboxysomes were described in *Ridgeia piscesae* symbionts (deBurgh et al., 1989), such inclusions, only known from microbes expressing

Form I Ribulose 1,5, bisphosphate oxygenase carboxylase (Rubisco) (Shively et al., 1998) were neither detected in the above mentioned species (Scott et al., 1994) nor in *R. pachyptila* (own unpubl. obs. M.B.) and the frenulates (Southward, 1982). This is in accordance to the expression of form II Rubisco in all investigated vestimentiferans (Robinson and Cavanaugh, 1995; Robinson et al., 1998).

The only sibloglinid in which a symbiosis with methane-oxidizing bacteria has been shown to exist is *Siboglinum poseidoni* from the Kattgat (Schmaljohann and Flügel, 1987). The methanotrophic bacterial symbionts involved are rod-shaped, gram-negative bacteria (Fig. 1h; Schmaljohann and Flügel, 1987). Conspicuous stacks of membranes arranged at various angles within the cytoplasm are present. Also membrane-bound vesicles, interpreted as lipid storage, have been found.

The complex vestimentiferan trophosome requires an elaborate blood vascular supply for the massive bacteriocyte tissue and its symbionts. The blood flows from the periphery towards the center of each lobule (morphological indications: van der Land and Nørrevang, 1975, 1977; Jones, 1988; live animals: Felbeck and Turner, 1995). A ramifying network of small peripheral blood vessels, connected with the ventral blood vessel of the trunk, spreads over the outer surface of the trophosome (Figs. 2, 3), thereby bathing the bacteriocytes' surface in fresh blood. The epithelium of each peripheral vessel opens and connects with the peritoneal sheath cells via apical junctional complexes. Thus, blood runs intercellularly between peritoneal cells, bacteriocytes, and myoepithelium, until it reaches the axial blood vessel that is connected with the main dorsal vessel. Bacteriocyte mitochondria with conspicuous electron-dense granules are mainly located adjacent to the intercellular blood sinuses.

The simple two-layered frenulate/*Sclerolinum* trophosome exhibits a blood vessel between the inner bacteriocyte epithelium and the outer peritoneal layer (Fig. 2 in Southward, 1982). Although not described in detail, the overall morphological organization of the trunk infers that fresh blood from the ventral blood vessel flows through the vessel of the trophosome to the dorsal vessel. Hence, the epithelial bacteriocytes are supplied with oxygen via their basal matrix.

The color of the adult trophosome in vestimentiferans ranges from light green to black. Judging from studies on *Riftia pachyptila*, the color variations are linked to the varying abundance of sulfur vesicles in the bacterial cells. Calculations of the number of sulfur vesicles per symbiont cell (Pflugfelder, 2003) as well as bulk measurements of elemental sulfur revealed that light green trophosome samples were connected with high elemental sulfur content, while black trophosomes contained low sulfur concentrations. These findings were explained by a variable sulfide supply in the environment. However, the

basic green color was hypothesized to come from inclusions in the trophosome peritoneum (trophochrome cells *sensu* Hand, 1987; green layer *sensu* Powell and Somero, 1986). This epithelium lacks in small juveniles and, accordingly, the color of the developing trophosome is white in all shades (unpubl. obs. M.B.). In larger juveniles and adults with a fully developed peritoneum, its green color appears to be overlain by the white sulfur content in the symbionts, thus giving the trophosome its typical color from light green to black (i.e. very dark green).

As mentioned above, *Rigeia piscesae* inhabiting different microenvironments, exhibits a 'short fat' and a 'long-skinny' growth type. Macroscopically, we studied some specimens without a visible trophosome (M.B. unpubl. obs.). Preliminary ultrastructural studies revealed that the bacteriocyte area was reduced to a few central bacteriocytes containing rod-shaped bacteria adjacent to degrading bacteriocytes (Fig. 1f).

Electron microscopy could not discover bacteria in the eggs of *Siboglinum poseidoni* (Flügel and Langhof, 1983; Langhof, 1987) or eggs and sperm of *Riftia pachyptila* (Cavanaugh et al., 1981; Cary et al., 1989; Jones and Gardiner, 1989). Neither PCR (polymerase chain reaction) amplification of the DNA from *Riftia pachyptila* eggs nor *in situ* hybridization with symbiont-specific oligonucleotides provided evidence for symbionts associated with gonads or freshly spawned eggs or sperm (Cary et al., 1993; Cary and Giovannoni, 1993). From both methodological approaches it can be concluded that the bacterial symbionts are not transmitted vertically from parent to offspring. Thus, in siboglinids evidence points to environmental transmission of the bacterial symbionts.

Uptake of symbionts is hypothesized to occur in early larvae, which still have an open gut, most likely from a free-living bacterial population. After an oral uptake, the bacteria are thought to be transported to the midgut where they become engulfed via phagocytosis by the endothelial cells (Southward, 1988; Jones and Gardiner, 1988, 1989; Callsen-Cencic and Flügel, 1995). Support comes from the finding of small sessile stages without a trophosome. Mouth, gut, and anus have been documented to be present in early juveniles of *Ridgeia piscesae* (Southward, 1988; Jones and Gardiner, 1988, 1989). Moreover, bacteria of unknown identity were detected in the lumen of the gut as well as in midgut cells (Southward, 1988; Jones and Gardiner, 1989). The authors interpret these bacteria as symbionts in primordial endodermal bacteriocytes. Mouth and gut are also present in early juveniles of *Siboglinum poseidoni*, but in this species an anus is lacking. Also here a few unidentified bacteria have been found located in vacuoles of midgut cells (Callsen-Cencic and Flügel, 1995). However, the characterization of these bacteria and their identity with the symbionts has not been scrutinized with molecular methods. It is possible that these larval bacteria represent just food items to be digested.

Functional considerations

All Pogonophora lack a functioning digestive system during most of their life. They rely on their thiotrophic (or methanotrophic in case of *Siboglinum poseidoni*) endosymbionts for their nutritional requirements (see Fisher, 1990; Childress and Fisher, 1992; Southward et al., 1986; Fisher, 1995; Nelson and Fisher, 1995). Transfer of organic carbon from the symbionts to the host occurs through digestion of symbionts (Bright et al., 2000) and through the release of small nutritive molecules consisting mainly of succinate and to a lesser degree glutamate (Felbeck and Jarchow, 1998). Activity of autotrophic processes in these symbioses highly depends on temperature (Childress et al., 1991; Scott et al., 1994), oxygen and sulfide concentrations (Fisher et al., 1989; Childress et al., 1991), nitrogen compounds (Hentschel and Felbeck, 1993), and elevated carbon dioxide concentration (Fisher et al., 1989; Childress et al., 1991).

Uptake of oxygen and hydrogen sulfide is facilitated through the anterior plume of the animals. This body region was found to be a very efficient gas exchange organ. Abundant extracellular hemoglobin in the blood and coelomic fluid binds with high affinity, simultaneously and reversibly to oxygen and sulfide (Arp and Childress, 1981, 1983; Childress et al., 1984, 1991). However, no sulfide was measured at plume level in the seep vestimentiferan *Lamellibrachia luymesii* (Julian et al., 1999). Also records taken at plume level of the 'long and thin' morphotype of *Ridgeia piscesae* showed very low concentrations of hydrogen sulfide (Urcuyo et al., 2003). In both species, the posterior part of the body is very long ('roots'). In *Lamellibrachia luymesii* this area was found permeable to sulfide (Julian et al., 1999) and uptake was demonstrated (Freytag et al., 2001). Also in *R. piscesae*, uptake through the hind end has been suggested (Urcuyo et al., 2003).

Molecular data on the symbionts

The symbionts of the majority of Vestimentifera have been studied, many of them from several locations (see McMullin et al., 2003). From molecular sequencing emerges the conclusion that only 4 strains or species of symbionts belonging to the subdivision of gamma-Proteobacteria are present (Nelson and Fisher, 2000; McMullin et al., 2003; but see Naganuma et al., 1997).

In brief, the Vestimentifera from hydrothermal vents share the same phylotype of symbiont (Edwards and Nelson, 1991; Feldman et al., 1997; Laue and Nelson, 1997; Nelson and Fisher, 2000, McMullin et al., 2003). Three clusters of seep symbionts are found in seep hosts. Depth appears to be defining which particular seep symbiont is present in a specific host (Nelson and Fisher, 2000; McMullin et al., 2003). Since the classification of these symbiont groups does not correlate with host classification, evolution of the symbiosis rather points to acquisition of local and habitat specific endosymbionts from the

environment. So far, no molecular data on the symbionts of Frenulata and *Sclerolinum* have been published.

Extracellular endosymbiosis in Olavius and Inanidrilus (Clitellata)

Within the annelid subtaxon Oligochaeta, two genera of marine Tubificidae, *Olavius* and *Inanidrilus*, are characterized by an obligate endosymbiosis with (mostly) extracellular bacteria. Since its first description (Giere, 1981), this association has been documented in more than 30 host species (see Table 1) and exists probably in all the approximately 80 species so far described belonging to these two monophyletic genera (Erséus et al., 2000, 2002; further material awaits description).

Not only is the number of closely related host species remarkable among marine Metazoa with prokaryotic symbionts, but also there is a wide variety of bacterial phylotypes associated with the gutless worms, be it in double, triple or even multiple symbiosis. This renders the model "oligochaete-bacterial symbiont" increasingly interesting for studies of co-evolution, routes of dispersal and modes of transmission. While the ease of access is an advantage compared to their gutless siboglinid counterparts, which mostly live in deep waters, the small meiobenthic size of *Olavius* and *Inanidrilus* poses problems to physiological studies.

The environment

Living in an environment where hydrogen sulfide is a regular ecofactor, the gutless oligochaete species are truly thiobiotic meiofauna (Giere, 1993), regularly exposed to low-oxygen and sulfidic conditions. They seem to prefer shallow sublittoral, calcareous sands of heterogeneous medium grain size typically occurring in depressions among coral blocks along the Caribbean and Australian shores. Here, they can develop rich stocks (*Inanidrilus leukodermatus* max. 100,000 ind. \times m⁻² in Bermuda) and an amazing diversity (18 spp. only in the Bahamas; Erséus, 2003). The amount of degradable detritus (mostly algal debris) in the sediment has to be high enough to allow for the establishment of a reduction zone in some depth underneath an oxic surface layer. Through their symbiosis with thioautotrophic prokaryotes (see below) which depend on the supply with hydrogen sulfide, occurrence of the host seems to be linked to the oxic/sulfidic interface. Thus, their prevalent occurrence could be experimentally positioned in the sediment column in relation to this threshold (Giere et al., 1991). Highly sulfidic (>500 μ mol H₂S) as well as purely oxic sands are usually avoided.

Table 1. Compilation of gutless oligochaete species with their multiple bacterial symbioses: Morphotypes assessed by ultrastructural, phylotypes by molecular biological methods.

Oligochaete host	Occurrence	Bacteria			Remarks for genetic identification
		Large, oval	Small, rod-shaped	Filliform	
<i>Inanidrilus leukodermatus</i>	Bermuda	γ	α		Dubilier et al. (1995); several subtypes each present (unpubl.)
<i>I. planus</i>	Bermuda	γ	α		Several subtypes of α present (Dubilier, unpubl.)
<i>Olavius longissimus</i>	Bermuda				
<i>I. bulbosus</i>	Belize				
<i>O. nicolae</i>	Belize				
<i>Inanidrilus</i> and <i>Olavius</i> , 11 more species	Belize				
<i>I. exumae</i>	Bahamas	γ	α		Dubilier et al., unpubl.
<i>I. triangulatus</i>	Bahamas	γ			Several subtypes of γ present (Dubilier et al., unpubl.)
<i>O. vacuus</i>	Belize or Bahamas?	γ	α		Dubilier et al., unpubl.
<i>O. finitimus</i>	Belize or Bahamas?				
<i>Inanidrilus</i> and <i>Olavius</i> , 9 more species	Bahamas				
<i>O. loisae</i>	Australia	γ	α	s	Dubilier et al. (1999); several subtypes of each present (unpubl.)
<i>O. albidus</i>	Australia	γ	α		Several subtypes of α present (Dubilier, unpubl.)
<i>Inanidrilus</i> and <i>Olavius</i> , 2-3 more species	Australia				
<i>O. crassitunicatus</i>	Off Peru	γ	δ	s	Several subtypes of each present (Dubilier, unpubl.)
<i>O. algarvensis</i>	Portugal				
<i>O. algarvensis</i>	Elba	γ	δ		Dubilier et al. (2001); several subtypes of each present (unpubl.); position mostly intracellular, fission longitudinal
<i>O. ilvae</i>	Elba	γ	δ		Dubilier et al. (2001); several subtypes of each present (unpubl.)

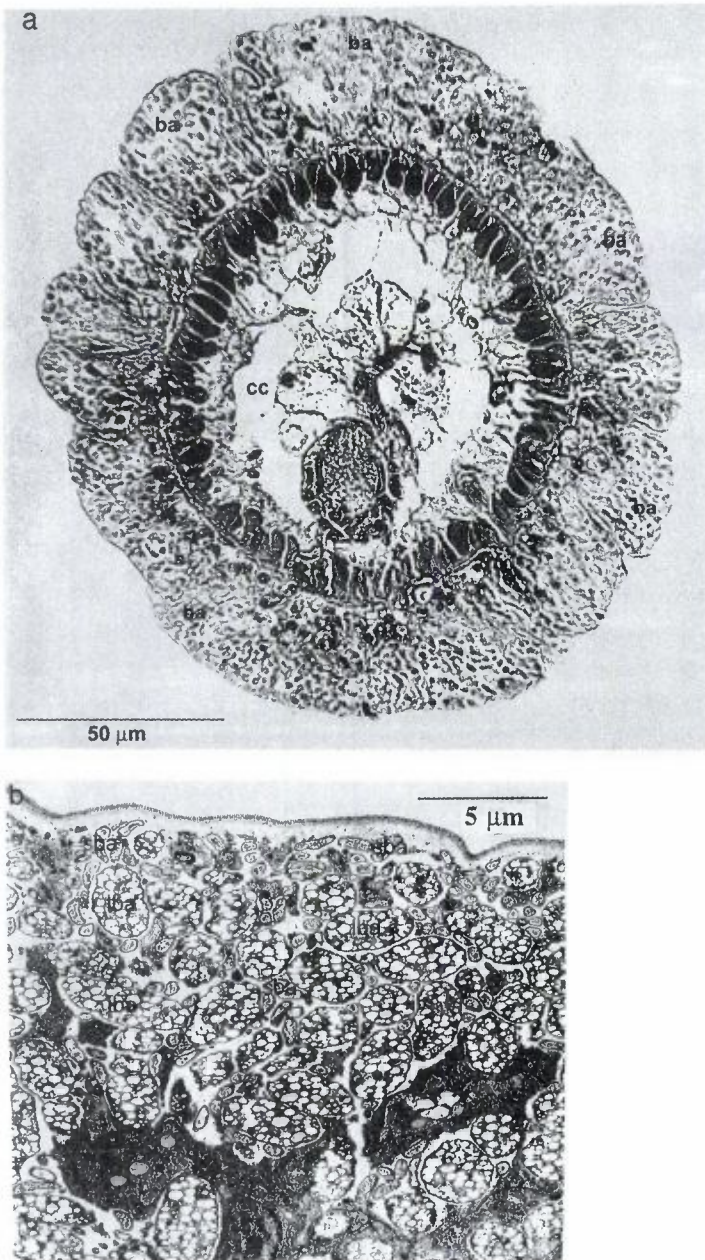


Figure 5. (a). Cross section through the gutless tubificid clitellate *Inanidrilus leukodermatum*. ba = subcuticular bacteria; cc = coelomic cavity containing ventral nerve cord and chloragogue tissue. Light micrograph. (b). Cross section through the subcuticular space of *Inanidrilus leukodermatum* showing two different bacterial morphotypes. Lba = large gamma-Proteobacteria; sba = small alpha-Proteobacteria. TEM micrograph (modified from Giere, 1989).

Recent studies, however, revealed exceptions from this generalized pattern of occurrence: *Olavius crassitunicatus* with its multiple phylotypes of bacterial symbionts including spirochetes (see below and Table 1; Giere and Krieger, 2001) was found at 300 m depth off the Peruvian coast in soft sediment covered by mats of sulfide oxidizing bacteria. On the other hand, *O. algarvensis* and *Inanidrillus ilvae* at the coast of the Island of Elba (Mediterranean Sea) were found in sediment with only traces of measurable hydrogen sulfide. Moreover, these species populated siliceous sand, so that also the mineral nature of the sediment does not seem to be a cogent factor for their occurrence. Compiling the distribution pattern, the numerous bacteria-symbiotic oligochaetes of *Olavius* and *Inanidrillus* are considered thiobiotic warm-water species with a rather euryoecious ecological background. This corresponds to their high variability of symbiotic partners (see below).

Morphology and ultrastructure of the symbiosis

From the initial TEM-studies, it was concluded that, as a basic pattern, all the *Inanidrillus* and *Olavius* species harbored two gram-negative bacterial morphotypes: large, oval bacteria dominating the subcuticular, intercellular space among extensions of epidermal cells and, mostly more peripheral, small rod-shaped or slightly bent bacteria (Figs. 5a,b).

Particularly the oval, larger bacteria were frequently found enclosed by epidermal cell extensions and compacted in large vacuoles and in various phases of lysis. Because of their regular presence in all the host species, the large oval prokaryotes were termed "primary" bacteria. In contrast to the situation in siboglinids, in the gutless tubificid annelids cell division was often found, albeit rarer so than in the small rods. The layer of bacterial symbionts underneath the cuticle has a specific arrangement along the worm's body. Absent from the anteriormost and posteriormost segments, the subcuticular bacteria become increasingly frequent from segment VIII backwards with maximum numbers in the postclitellar region. In the genital segments XI to XIII, the bacteria become aggregated in a conspicuous ventral "genital pad" which surrounds the ovipores while in this region the dorsal and dorsolateral parts of this body region are almost devoid of bacteria.

However, the general pattern described above had variations in the size of the bacteria (e.g. the large, oval ones ranged in length from 2.0 μm in *Olavius finitimus* up to 7.3 μm in *O. crassitunicatus*), and in their shape (e.g. in the smaller morphotype from rods to croissant-shaped bacteria). More striking were structural variations in details within the same host species: In *O. algarvensis* from Portugal the large bacteria are extracellular and divide by cross-section, while in the same host species from the Island of Elba they are almost always found enclosed in vacuoles of the epidermal cells and their division is by longitudinal fission.

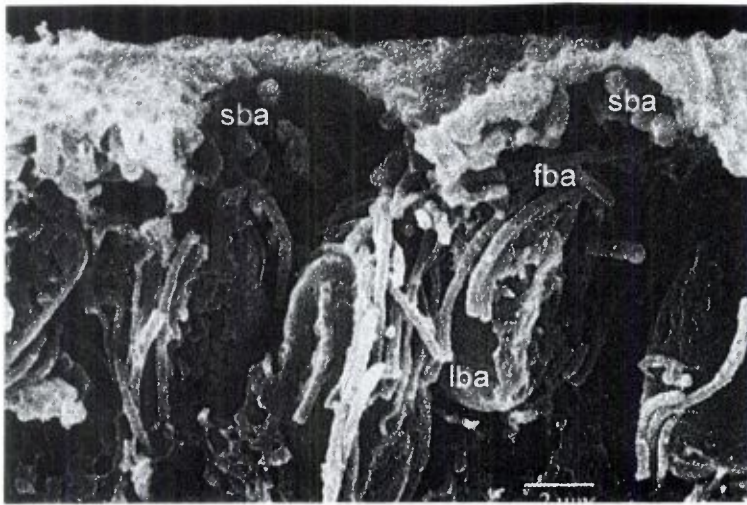


Figure 6. *Olavius crassitunicatus* with cuticle peeled off showing three bacterial morphotypes in subcuticular space. Fba = filiform bacteria. SEM micrograph. (Original O.G.).

Moreover, the overall number of the symbiotic prokaryotes and the thickness of the "bacterial layer" in the host's body is much reduced. The well-developed body musculature encloses a considerably reduced lumen of the normally wide coelomic cavity (Giere and Erséus, 2002). In other host species, more than two bacterial morphotypes are clearly discernable. In *Olavius loisae* from Australia a third prokaryotic type with a tortuous cell wall did not fit into the usual pattern, in *O. crassitunicatus* from the coast of Peru a new bacterial morphotype was present: sinuous filaments indicative of spirochaetes (Fig. 6).

Considerations on the evolution of this obligate and multiple symbiosis in a large species group are necessarily linked to the mode of symbiont transmission. The regular occurrence of the large, oval bacteria in all the symbiotic species and structural analyses of *Inanidrilus leukodermatus* from Bermuda in the phase of egg deposition indicated that this bacterial morphotype was transmitted from the parental host generation to the offspring in a modified vertical pathway (Giere and Langheld, 1987): As a generalization from several host species it can be concluded that, in contrast to all other Oligochaeta, gutless, bacteria-symbiotic tubificids do not deposit their eggs in a cocoon produced by clitellar secretions. Instead, they are laid singly onto the sand grains where they adhere with their sticky mucous surface. The large egg is pressed through small ovipores, which are directly surrounded by the "genital" or "ventral pad". Confirmed by observations in culture experiments, it is

contended that the thin cuticle of the genital pad, densely packed with bacteria, would then rupture and the bacteria would be released onto the surface of the egg (Giere and Langheld, 1987; Krieger, 2001). In embryonic worms, a rich number of prokaryotic cells was found enclosed in vacuoles in the epidermal cytoplasm.

Through molecular analysis combined with immunohistological labelling we could genetically identify the released bacteria "infecting" the egg as direct descendants of the large, oval prokaryotes (Krieger, 2001). In the parental host, they initially aggregated at one pole of the spindle-shaped egg underneath the mucous cover and the egg membrane. The symbionts then penetrated the egg membrane and invaded the blastomers where some of them were lysed, but others seemed to further develop and divide (Krieger, 2001). There was no evidence for transmittance via the ovaries or the maturing eggs.

While the transmittance of the large bacterial "primary" morphotype could thus be clarified, the pathway of the smaller rod-shaped "secondary" bacteria remains open. The genetic variance in the different oligochaete hosts (see below) points to a horizontal or even environmental transmittance, in any case a less direct mode. In evolutionary terms, this would leave open the window for uptake of a variety of bacteria.

Functional considerations

Complete reduction of the alimentary and nephridial excretory system proves the obligate nature of the bacteria-symbiosis evolved in these gutless oligochaetes. Already the thickness of the bacterial layer, representing a calculated 25% volume of the host's body and consisting of almost 1 million bacterial cells per worm (Giere et al., 1995), underlines the relevance of this symbiosis. This is corroborated by the regularity of this pattern in all of the species studied.

Their distributional pattern, preferably in low oxic to mildly sulfidic subsurface layers of sand, is otherwise unknown in interstitial annelids and indicates a relation of the symbiosis to hydrogen sulfide. This was corroborated by the regular presence of several membrane-bound globules in the cytoplasm of the larger "primary" symbiotic bacteria and favored the assumption that this morphotype might be a sulfide oxidizer. The content of the globules is probably an oxidation product, a viscous polysulfur, which frequently represents an energy storage product easily to be activated in cases of shortage of external reduced sulfide. Uptake experiments pointed to a preferred oxidation of non-toxic thiosulfide over the toxic hydrogen sulfide (Giere et al., 1988b).

The bacteria deposit their metabolic energy reserves as polyhydroxybutyrate stored in numerous non-membrane-bound globules in the cytoplasm (see Fig. 5b). It is the refringence of incident light by these bacterial globules that render their hosts the characteristic shiny white appearance. It

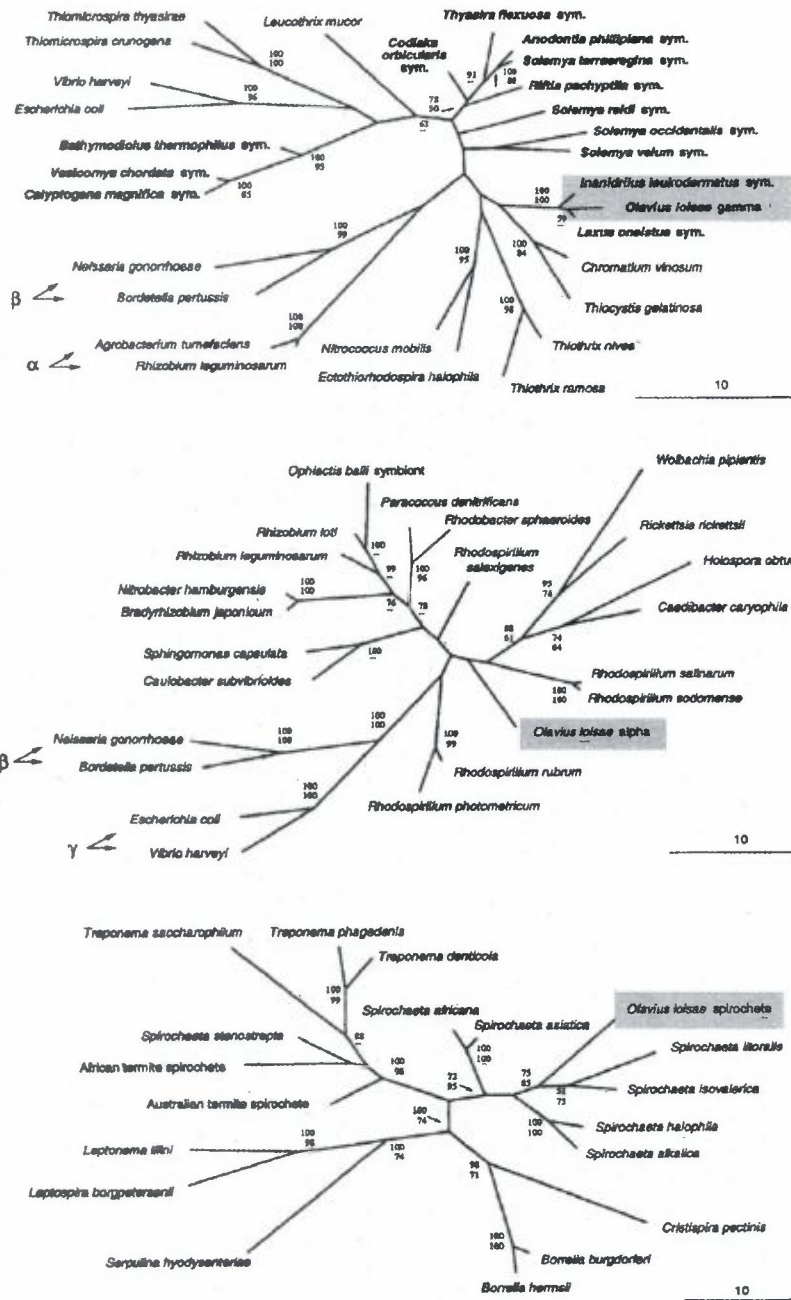


Figure 7. Phylogenetic trees with the symbionts in Annelida highlighted (from Dubilier et al. (1999) modified).

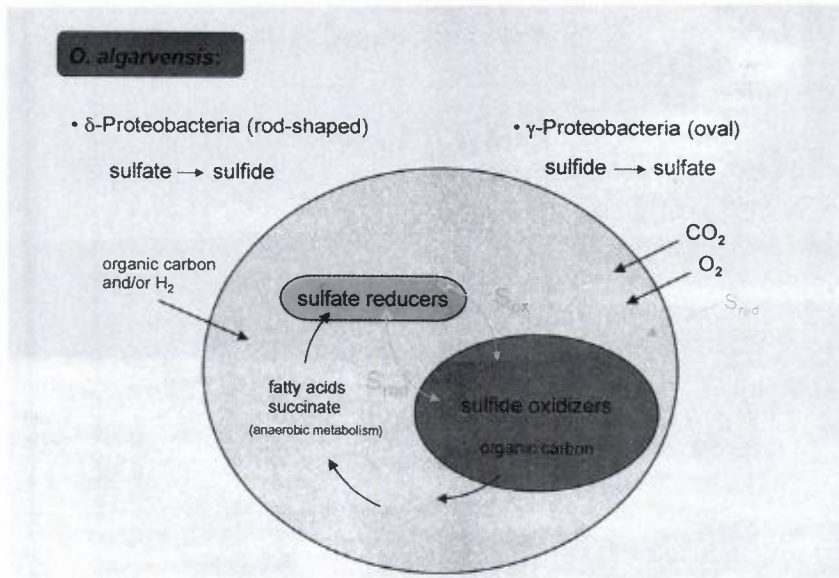
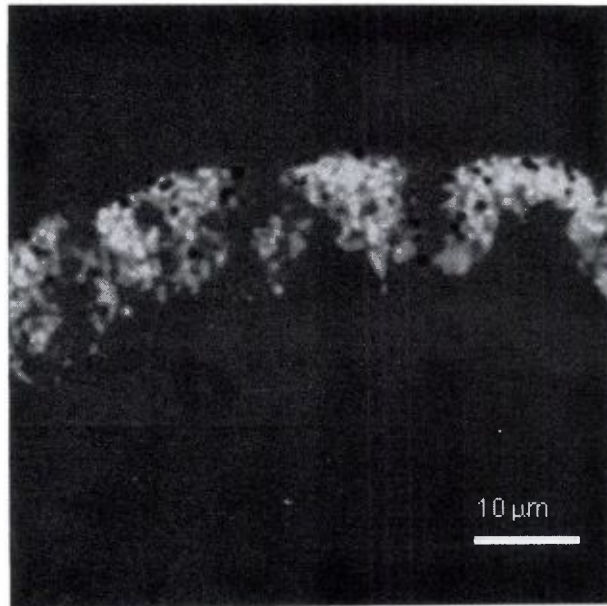


Figure 8. (a). Cross section through *Olavius algarvensis* from the Island of Elba (Italy) showing different FISH fluorescence of two kinds of Proteobacteria: in peripheral position directly underneath cuticle (original: red) delta Proteobacteria; bulk of fluorescent bacteria between cuticle and epidermis in more central position (original: green) gamma Proteobacteria. Light micrograph. (b). Scheme of syntrophic relations between two bacterial phylotypes in *Olavius algarvensis* from the Island of Elba (Italy) (from Dubilier et al. (2001) modified).

turns into a pale grey after extended periods without supply of new hydrogen sulfide. The combination of ultrahistochemical and molecular methods using immunogold-label proved the presence of chemoautotrophy and sulfur metabolism in the large bacteria of all the host species tested (Krieger et al., 2000). On this basis, supported by molecular data (see below), it is concluded that the large bacteria are, in fact, oxidizing reduced sulfur compounds.

Molecular data and reconstructions of phylogenetic trees corroborated the function of the gamma-Proteobacteria in oligochaetes as oxidizers of reduced sulfur species, related to free living sulfur oxidizers (Fig. 7). The close phylogenetic relationship to the epibacteria of thiobiotic nematodes such as *Laxus* should be mentioned in this context.

The nature and function of the heterogeneous group of small rod shaped bacteria, remains unresolved through structural or chemical analyses. Absence of all nephridia in the hosts, marked nitrogen peaks in the bacteria and lack of any trace of sulfur (elemental electron spectroscopy) let us assume a relation to the nitrogen metabolism of the worms, but this awaits molecular scrutiny (see below). The position of the alpha-Proteobacteria in the trees does not allow a functional conclusion, although the taxonomic relationship to various bacteria metabolizing nitrogen compounds (Dubilier et al., unpubl.) would support earlier assumptions of a role in the nitrogen cycle of the anephridial annelid hosts.

While the nature of the third morphotype of prokaryotes could be deduced from its filiform, tortuous shape, its symbiotic function remains unclear. Spirochaeta are often pathogenic, but the annelid symbionts are related to free-living marine sprochetes. Their regular occurrence in several host species without any malformations of cells or tissues let us assume a mutualistic or at least non-deleterious association. The function of the Spirochaeta in the multiple symbioses cannot yet be assigned.

In one host species, *Olavius algarvensis* from Elba, the functional role of the dual symbiosis (gamma- and delta-Proteobacteria) could be evidenced. Molecular analysis combined with fluorescence *in situ* hybridization (FISH) using specific probes revealed that the delta-Proteobacteria reduce sulfate to sulfide or another reduced sulfur compound which is then excreted and taken up by the adjacent gamma-Proteobacteria. They, in turn, gain metabolic energy from the oxidation of these reduced sulfur species producing sulfate with oxygen as an electron acceptor. This interactive function of the two bacterial partners results in a "syntrophic" pathway, which would enable the hosts to become rather independent of a regular supply of hydrogen sulfide (Fig. 8a,b).

Summarizing the dual, triple or multiple bacterial symbioses in the numerous oligochaete species and considering their diversity in closely related or even conspecific tubificids, we conceive a complexity unique in annelids. From the restriction of these obligate symbioses to a derived monophylum with only two

genera within the phallodriline Tubificidae (Erséus et al., 2002), and from their worldwide occurrence and high number of host species, we can infer important ecological and phylogenetic conclusions:

The acquisition of bacteria as a relatively recent event with their following internalization into the body plan of these worms caused a cascade of major structural, physiological, ecological and evolutionary steps: In order to become symbionts of value to the hosts, the bacteria were "domesticated", controlled in their multiplication (cell fission in the symbionts seems to be rather rare compared to free living bacteria), and regulated in their arrangement in the body structure (both along the length of the body as well as their embedding into the cuticle/epidermis complex only). Before abandoning basal organ systems like the digestive and excretory tract, a secure mode of symbiont transmission has to be established and metabolic pathways modified and intimately fine-tuned to suit the array of symbiotic partners. The metabolic needs of the symbionts have altered the ecological demands and vertical distribution of their hosts (Giere et al., 1991). While other biological domains such as the complicated hermaphroditic reproduction remained apparently unchanged, one trait of possible relevance for the incorporation of the bacteria was altered: These oligochaetes deposit their eggs freely onto the sand grains rather than enclosing them in cocoons. This feature, unique in clitellates, may be interpreted as a step opening a window for environmental bacterial transmission (see above). This would allow for flexibility of the symbiotic pattern with a high adaptive potential and would favor the wide distribution of the symbiosis under different ecological and climatic conditions.

The ancestral host and the early developmental steps in this symbiosis are, as yet, unknown. Discovery of symbiotic host species having retained a reduced or vestigial intestinal system and/or associated with just one bacterial phylotype would be of great relevance. Are all the different symbioses we have studied today the result of one event integrating bacteria into the biology of a phallodriline ancestor or did the uptake happen independently several times and at several places? Also not yet reconstructable are the zoogeographic pathways along which these oligochaetes without any swimming larvae spread their new "invention of a bacterial symbiosis".

Doubtlessly this represented a "quantum leap" for the gutless oligochaetes. In shallow water environments with their unstable life conditions a variety of bacterial symbionts might be advantageous over just one single symbiont and better enable the host responding to environmental variations. On the other hand, in multiple symbioses competition between symbionts for resources and space is more complex and regulation is more difficult. In this scenario, sequential or cooperative uptake of metabolic products and environment-derived use of resources would decrease competitive pressure and favour symbiont diversity (Dubilier, 2003). In any case, the high species number

within only two oligochaete genera and the intensive modification of their symbiotic pattern indicates the ongoing of a rapid radiation and successful evolution.

Molecular data on the symbionts

The diversity of morphotypes in the bacterial symbionts of gutless oligochaete species, evidenced above, is exceeded even by that of the phylotypes emerging from molecular genetic analyses (Table 1). The large, oval bacteria belong to the gamma subclass of the Proteobacteria. They form a cluster of closely related species (3–5% genetical divergence; Dubilier, 2003) in the vicinity of free-living sulfur oxidizing bacteria. In contrast, the small rods are genetically rather heterogeneous: Representing in some hosts alpha-Proteobacteria, in others they fall within the delta-Proteobacteria. The filiform bacteria, the tertiary morphotype, have been characterized as Spirochaeta in two host species and will probably be found in additional host species (*O. finitimus* seems a good candidate).

The phyletic positions of the various symbiotic bacteria is not yet complete enough to reveal a pattern of evolutionary lines along which the symbioses might have developed and diversified. Nor do we know the phylogenetic background of the host (Erséus et al., 2002) thoroughly enough to link evolutionary with zoogeographical pathways in a possible co-evolution between host and bacterial symbionts.

Ectosymbiosis in Alvinella (Polychaeta)

Alvinellid polychaetes (Terebellida), known only from deep-sea hydrothermal vents of the Eastern Pacific, can dominate with their thickets of tubes entire communities of vent biota. *Alvinella pompejana* and *A. caudata* are regularly found associated with epibacteria. In contrast, representatives of *Paralvinella* seem to lack this episymbiosis (Cary et al., 1997; Desbruyères et al., 1998 and literature therein). The variety of bacterial types involved, the different autapomorphies of the worm's body related to its epibacteria, and the extreme environment of *Alvinella* render this symbiosis complex and functionally still not resolved.

The environment

The species name, *pompejana*, relates to the unique environment of the polychaete genus *Alvinella*: It regularly lives in a rain of mineral particles from hot, active sulphide-expelling chimneys and smokers in many vent areas of the East Pacific Rise. For *A. caudata* essentially the same geographical distribution (21°N to 17°S) and habitat is recorded.

In situ sulfide measurements revealed that the environment of *Alvinella pompejana* has very high concentrations of hydrogen sulfide, unusual even for the sulfide biota of the East Pacific Rise vents. Measurements of 300 μM (Sarradin et al., 1998) and 1,520 μM at 13°N Genesis (Le Bris et al., 2003) and 350 μM 9°N (Luther et al., 2001) are not uncommon and surpassed by recent recordings in the millimolar range (Le Bris, 2003).

Early on, Chevaldonné et al. (1992) observed temperatures above 100°C in the vicinity of *A. pompejana* that appeared not to affect the animals. More recent *in situ* temperature measurements in the environment ranged from above ambient seawater up to 250°C (Sarradin et al., 1998; Luther et al., 2001; Le Bris et al., 2003). Temperature measurements up to 110°C in the tube thicket (Cary et al., 1998) corroborated previous findings and led Cary et al. (1998) to hypothesize that this animal lives at body temperatures well above 60°C. However, Chevaldonné et al. (2000) seriously doubt these high values. The actual temperature in the body is probably only 30–35°C with fluctuations up to 50°C.

Morphology and ultrastructure of the symbiosis

In *Alvinella pompejana*, the pinkish to red body surface has a fuzzy appearance due to unique, histologically highly specialized "bacteriophores", digitiform or hair-like protuberances of one cm length. They bear the main portion of the bacterial biomass (Fig. 9). These protuberances are dorsally arranged in transverse rows and laterally positioned between the noto- and neuropodium. They are not covered by a normal cuticle but bear a microvillar apical surface that secretes globular mucus drops with a reticulated, somewhat fibrous appearance. Embedded in this matrix are the terminal ends of numerous filiform bacteria, longer ones (10 μm \times 1 μm) at the tips of the protuberances, shorter ones at their basal parts. These bacteria form a dense whitish felt covering the dorsum and, laterally, the proximal parts of the parapodia. Filamentous bacteria also arise from the worm's posterior parapodia. In the intersegmental grooves grow filiform bacteria together with rod-shaped and coccoid bacteria in clusters of irregular composition. In addition, the entire worm's tegument is colonized by several bacterial morphotypes (sheathed or unsheathed filaments, spirally-shaped threads, rods) giving the worm an overall fuzzy, hairy appearance.

In the little studied congener *Alvinella caudata*, only the tapering posterior part of the body bears filamentous epibacteria, which arise from special irregular digitations of the enlarged notopodia. Both species have a well developed and functional digestive system. The gut content has been found to consist of bacterial remains mixed with a high portion of polymetallic spherocrystals giving high signals of sulfur.

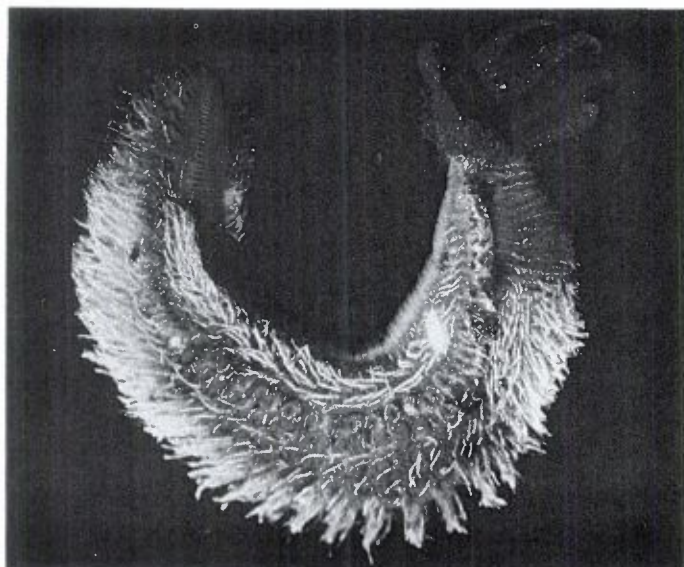


Figure 9. Micrograph of *Alvinella pompejana* with ectobacterial symbionts.

Functional considerations

What is the nature of this annelid/bacteria association? Although not confirmed by direct observations or measurements, Desbruyères et al. (1998) stated that the epibacterial consortia of *A. pompejana* as well as those from the inside of its tube "might be involved in the nutrition or detoxification of the host. This reasoning would support their characterization of this symbiosis as a complex and obligate symbiosis, "remarkable due to its magnitude and anatomical patterns".

The modifications found on those segments of *Alvinella* that carry bacteria are evidence for a close relationship between the microbes and their host. The specific nature of the association – be it nutritional or protective by sulphide detoxification – has yet to be elucidated (Alayse-Danet et al., 1987). A protective effect by the bacterial partners against the noxious impact of heavy metals and sulfides in the ambient seawater seems rather questionable regarding the large surface of the worm with numerous diffusive pathways into the body and the inevitable uptake of toxicants via nutrition (see Dubilier et al., 1997; Windoffer et al., 1999).

It is unclear to what extent *A. pompejana* feeds on its epibiotic bacteria. While stable isotope data point to a bacterial food source (Desbruyères et al., 1983), behavioral observation and gut content analysis rather suggest grazing on

the tube as the usual mode of nutrition in this worm. The biology of *A. pompejana*, which is an early coloniser of vent chimneys and has a remarkably high temperature tolerance, has been comprehensively summarized by Desbruyères et al. (1998).

Molecular data on the symbionts

Although uptake of inorganic carbon and RuBisCo activity have been reported (Alayse-Danet et al., 1986), their low levels make an important contribution of autotrophs unlikely. The presence of bisulphate reductase genes in bacteria suggests that they are involved in the anaerobic sulphate-reduction (Cottrell and Cary, 1999), a general process characteristic of the typical "white smokers" frequently colonized by *Alvinella* spp. (Baross and Deming, 1995).

Molecular analyses in alvinellids are restricted to samples of filamentous bacteria cut off from the dorsal hair-like expansions of *A. pompejana* or from parts of its dorsal tegument. According to Haddad et al. (1995) the diverse epibacterial consortium consists mainly of four clones of epsilon-Proteobacteria, while Cary et al. (1997) differentiated only two main phylotypes of heterotrophic epsilon-Proteobacteria, one of which was common also on various surfaces in the environment. The other type colonized the dorsal appendages of both *A. pompejana*, and *A. caudata*. Campbell and Cary (2001) additionally demonstrated the presence of two types of Spirochaeta on *A. pompejana*, but found these types also to occur in various high-temperature areas, which questions their symbiotic relevance.

Campbell et al. (2001) deduced from isolations in enrichment cultures the existence of two functional groups among the bacteria on the hair-like projections: (1) moderately thermophilic sulfur reducing heterotrophs, and (2) lithoautotrophs which grow on sulphur using H^+ as electron donors (optimal temperature 41–45°C).

In contrast, the cluster-like associations of non-filamentous bacteria growing in the intersegmental spaces showed strong incorporation of autoradiographic label following *in situ* incubations of *A. pompejana* with ^{14}C -bicarbonate (Alayse-Danet et al., 1986). This would characterize them as autotrophic.

In contrast to Desbruyères et al. (1998), Cary et al. (1997), and Campbell and Cary (2001) postulate no obligate requirement of *Alvinella* to live associated with bacteria. They define this bacterial community as 'symbiotic in the most general sense' resembling a "cohabitation of dissimilar organisms". Compared to the intricate endosymbiotic relations of siboglinid and tubificid annelids, the connection of the epibacterial association to the alvinellid annelids is rather loose, but it needs further scrutiny.

*Lesser known symbioses**Xenonerilla bactericola* (Polychaeta)

The nerillid *Xenonerilla bactericola* lives in dysoxic sediments of the Santa Barbara basin off California. A single morphotype of small bacteria covers the host (Müller et al., 2001). The habitat suggests a thiotrophic physiology of the microbes. However, the nature of the symbiosis has yet to be determined.

Tubificoides benedii (Oligochaeta)

An occasional association with epibacteria was observed also in the marine oligochaete *Tubificoides benedii* (Dubilier et al., 1995; Giere et al., 1988a) and in other freshwater Tubificidae (unpubl. observ. O.G). Occurring in sulfidic muds of North Sea tidal flats or in muds of a thermomineral, sulfidic stream in Romania, these macrobenthic worms bear a coat of whitish filiform bacteria on their hind end. The intimate connection of the bacterial threads with insertions of the epidermal cells indicates a well-organized interrelation between prokaryote and metazoan.

Molecular studies revealed that the epibacteria of *T. benedii* belonged to the gamma-Proteobacteria (Dubilier et al., unpubl.) and were closely related to the thiotrophic endosymbionts of *O. algarvensis* from Elba (see above). Although this points to an uptake and oxidation of the abundant environmental sulfide, the protective relevance of this bacterial cover against the influx of toxic hydrogen sulfide seems limited (Dubilier et al., 1997).

3. Symbioses in Annelids Unrelated to a Chemosynthetic Environment

Acidovorax nephridial symbiosis

In Lumbricidae (Oligochaeta) the paired metanephridia, present in each segment, harbor extracellular endosymbionts. Each nephridium consists of a tube with three major loops. A bacterial biofilm is consistently found in the widening of the second loop, the ampulla (Fig. 10) (Knop, 1926; Pandazis, 1931; Buchner, 1965; Scott and Musgrave, 1971; Villaro et al., 1985).

16S rRNA sequences of these nephridial bacteria of *Lumbricus terrestris*, *Aporrectodea tuberculata*, *Octolasion lacteum*, and *Eisenia foetida* revealed that the symbionts form a monophyletic cluster within the genus *Acidovorax* (beta-proteobacteria) (Schramm et al., 2003). The association appears to be specific since no other bacteria were detected in the nephridia using an *Acidovorax*-specific oligonucleotid probe and various group-specific probes for fluorescence *in situ* hybridization (Fig. 9). Symbiont sequences obtained from

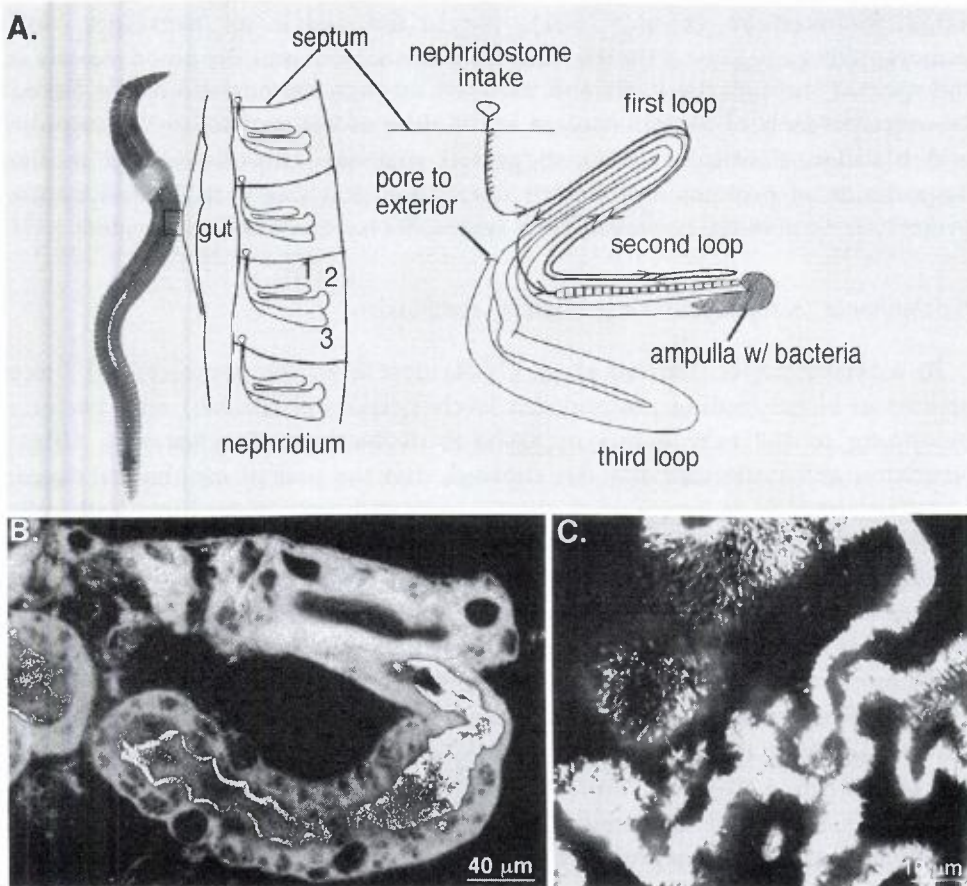


Figure 10. Bacteria in the nephridia of the common compost worm *Eisenia foetida*. (a). Diagram illustrating the location, anatomy and direction of flow for the earthworm nephridium. The three loops of the nephridium are labeled, with bacteria in the ampulla of the second loop. (b). Cross-section through the ampulla showing the *Acidovorax* sp. bacterial symbionts labeled by *in situ* hybridization with and *Acidovorax*-specific oligonucleotide probe. (c). Enlargement of cross-section through B, illustrating the bacteria adhering to the folds of the nephridium.

several specimens of the same host species show a high similarity. This suggests a highly specific host-symbiont interaction and possibly points to co-speciation of host and symbiont (Schramm et al., 2003).

The function of the *Acidovorax* nephridial symbiosis is not well understood. Closely related free-living freshwater *Acidovorax* aggregations are proteolytic and found to degrade organic matter releasing dissolved amino

acids (Schweitzer et al., 2001). Nephridia serve as excretory and osmoregulatory organs. Filtered fluid from the coelom and the blood vessels is transported through the tubes and excreted through the nephridiopore. Based on measurements of protein content in the fluid of the coelom, in the ampulla and bladder, Pandazis (1931) suggested that the ampulla serves in the degradation of proteins and further speculated that this is facilitated by the proteolytic symbionts. However, such symbionts have yet to be cultivated.

Reichenowia oesophageal (mycetomal) symbiosis

In a recent paper, Siddall et al. (2004) described the symbiosis of three species of blood-feeding placobdellid leeches (Glossiphoniidae) with bacteria belonging to the new genus of alpha-Proteobacteria, *Reichenowia*. Ultrastructural and molecular analysis showed, that the pair of esophageal caeca, known since long as 'esophageal glands' or 'mycetomes', were lined with cells packed with intracellular rods of somewhat varying size. Genetic analysis revealed that the bacteria in each of the host species were unispecific, but closely related. They grouped separately in the vicinity of the Rhizobiaceae, bacteria involved in the nitrogen metabolism of plants. Observations of mycetomal cells releasing the bacteria into the lumen of the esophagus and the absence of mycetomal organs in non-haematophagous glossiphoniids justify the assumption that the bacteria have a digestive function breaking down the ingested blood, a situation similarly found in other bloodfeeding ectoparasites. Transovarial, direct transmission of the bacteria is suggested. Other haematophagous leeches probably have corresponding bacterial symbioses.

Rickettsia epidermal/oesophageal/intestinal symbiosis

The digestive system (oesophagus and intestine) and epidermis of the glossiphoniid leech *Toris tagoi* harbor *Rickettsia*-like intracellular bacteria (Kikuchi et al., 2002). An electron translucent halo surrounds each bacterium and possibly points to a (non-well preserved) symbiosome membrane.

In a diagnostic PCR analysis using universal and specific primers several glossiphoniid species were screened for Rickettsiales (alpha-Proteobacteria). *Toris tagoi* and *Hemicrepsis marginata* were *Rickettsia* positive, while in 7 other species neither *Rickettsia* nor the closely related *Wolbachia* were detected. The symbionts were found in the majority of *T. tagoi* specimens (97.7%) and in all *H. marginata* specimens investigated. 16S rRNA sequences and phylogenetic analysis confirmed the presence of *Rickettsia* in these two species. Furthermore, the presence of symbionts in some of the hatchlings brooded by one specimen of *T. tagoi* was taken as an indication of vertical

transmission. The symbiont genus could be divided into two monophyletic groups, one associated with leeches, the other one associated with arthropods (Kikuchi et al., 2002).

Rickettsia species are obligate endosymbionts (Weisburg et al., 1989) of blood-sucking terrestrial arthropods (Chen et al., 1996; Davis et al., 1998; Fukatsu and Shimada, 1999; Noda et al., 1997; Werren et al., 1994) or leeches (Kikuchi et al., 2002). While many *Rickettsia* species from arthropods are non-pathogenic, some facultatively can infect humans or other vertebrates and act as disease vectors, for example causing spotted fever or epidemic typhus (Dasch and Weiss, 1992). Analogous to the terrestrial bloodsucking arthropod symbiosis, it was suggested that the aquatic bloodsucking leeches occasionally also cause diseases in amphibians and fish (Kikuchi et al., 2002).

4. Conclusion and Outlook

Annelids have developed repeatedly and independently all kinds of symbioses with bacteria. In loose and apparently occasional associations the worm's body perhaps serves as a favorable anchoring substrate only (e.g. the tubificid oligochaete *Tubificoides benedii*) while in the alvinellid polychaetes from hot vents the association with epibacteria on specialized surface structures of the body seems somewhat more elaborated. The obligate association with various phylotypes of endobacteria in gutless oligochaetes (Tubificidae), although just filling the extracellular subcuticular space, is evidently more intimate allowing the hosts to give up major organ systems. The specific pattern of bacterial cell arrangement in the body is clearly regulated, as is the mechanism of transmission, which seems to be a complex and evolutionary favorable combination of vertical and environmental processes. It is in the annelid family Siboglinidae that the major step towards an obligate intracellular symbiosis has been realized resulting in the formation of a central massive organ, the trophosome, in intimate contact to a morphologically and physiologically specialized blood system.

The occurrence in a rigid sulfidic environment indicates an adaptive value even of an occasional and superficial association of annelids with bacteria (tubificid oligochaetes with a coat of sulfur bacteria). Similar to their interstitial meiobenthic gutless relatives, the speciose genera *Olavius* and *Inanidrilus*, all of these worms can live in a sheltered environment of reduced competition and ample nutrition rich in dissolved organism substances and bacteria. Through the bacteria, new trophic sources are made available to the annelid hosts inhabiting seeps and vents. They enable these annelids to thrive in chemically exotic environments with sulfide and methane prevailing. Combined with a high resistance for heavy metals and elevated temperatures,

these bacteria symbioses open up settlement in biotopes considered hostile for life of higher animals.

A trophic specialization, blood feeding, probably related to the establishment of highly evolved bacteria symbioses in leeches and seems established several times (independently?) in the group of Hirudinea.

Annelid-bacteria symbioses are found in singular species, in a radiating species group consisting of just a few genera, or in the entire set of higher taxa (families or orders). This indicates a different evolutionary age and a differently deep structural and physiological impact on the organisation of the host. Annelids, as other animal taxa, have apparently repeatedly established a symbiotic association of adaptive significance. Exemplified in the huge tube worms *Riftia* and its relatives, this symbiosis can be considered the characteristic step towards establishment of many hydrothermal vent and seep communities.

Future research will have to concentrate on physiological details, evolutionary trends and distributional pathways in these symbioses. Despite the challenge of the exotic, extreme and often unknown biotopes, research in the deep-sea world is severely hampered by logistic demands, physical constraints and experimental problems. With respect to this drawback, the shallow water gutless oligochaetes are more suitable symbiotic models. Work with these interstitial annelids, however, is cumbersome due to their meiobenthic small size.

Yet, the decisive obstacle for significant progress in research on the evolution and physiology of these symbioses, including their ecological consequences, is probably the impossibility to cultivate the bacteria in isolation and to perform re-infection experiments with their host animals, which were made aposymbiotic. In this respect, the ectobacterial symbiotic models are clearly favoured.

Despite these difficulties, further studies on polychaete and clitellate symbioses could render valuable contributions to profound biological problems:

- reductive ontogenesis in basal levels of entoderm development
- regressive evolution of organs,
- phylogenetic impact resulting from a symbiosis,
- development of new animal designs structured by symbiosis
- colonization of exotic environments
- utilization of novel trophic resources.

Thus, beyond the scope of just annelid research the understanding of annelid symbioses could become milestones of general significance.

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