Helgol Mar Res (2003) 57:139–143
DOI 10.1007/s10152-003-0152-4

ORIGINAL ARTICLE

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Bacterial community in the tunic matrix of a colonial ascidian *Diplosoma migrans*

Received: 5 March 2003 / Revised: 30 April 2003 / Accepted: 30 April 2003 / Published online: 13 June 2003

Abstract This paper provides the first information on the morphology of different morphotypes of bacteria in the tunic matrix of the colonial ascidian *Diplosoma migrans*. Ascidians were collected from waters near Helgoland (German Bight, North Sea). The dominant type is represented by extremely high numbers of long, needle-like rods (length 10–30 μm, width 0.5 μm). The bacteria are motile by means of bipolar monotrichous flagella, generating swift sigmoidal movement. Bacteria are already present during different embryonic stages. It is assumed that they are transferred during sexual propagation from the parental colony to its offspring. As a second morphotype, the tunic harbors screw-like bacteria in low numbers (length 4–10 μm, width 0.5 μm). Besides these conspicuous morphotypes, occasionally motile rods with spore-like globules at one end and additional coccoid forms in large quantities of unknown meaning (possibly spores) were found. The taxonomic status and ecological functions of these differently shaped bacterial groups are unclear.

Keywords Ascidiacea · Bacteria · Didemnidae · Tunic · Tunicata

Communicated by H.-D. Franke

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Introduction

Symbiosis research is a fascinating ecological field of growing interest. The elucidation of specific strategies of interactions in communities opens new insights into the multifarious forms of marine life. Studies of pro- and eucaryotic interactions previously focused on terrestrial organisms (Görtz and Maier 1991; Heckmann and Görtz 1992). However, the number of reports on interactions between bacteria and the wide scope of marine organisms has been increasing (Fenical et al. 1991). Bacteria have been detected in microalgae (Kirchner et al. 1997, 1999, 2002; Kopp et al. 1997; Seibold et al. 2001), in corals (Paul et al. 1986), in annelids (Cary et al. 1997), in echinoderms (Deming and Colwell 1982; Roberts et al. 1991; Burnett and McKenzie 1997), in cnidarians (Palincsar et al. 1989), in sponges (Althoff et al. 1998; Perovic et al. 1998; Prokic et al. 1998), and in other taxa. However, phylogenetic relationships, ecological functions and the biochemical background of epibiontic or intracellular bacteria have mostly remained unclear. There are only a few reports on bacteria associated with ascidians. Hirose and Saito (1992) detected corkscrew-shaped motile bacteria in the tunic matrix of the tropical *Botrylloides simodensis*. Hirose et al. (1996) also described bacteria of identical morphology from the tunic of the bioluminescent *Clavelina miniata*. The authors concluded that bioluminescence may not be attributed to bacterial activities. In the colonial ascidian, *Lissoclinum punctata*, Hirose et al. (1998) observed the symbiotic photosynthetic *Prochloron* sp. inside and outside the tunic, and Mackie and Singla (1987) detected bacteria in the tunic of *Diplosoma listerianum* and *D. macdonaldi*. For the benthic, colonial ascidian *D. migrans* of the North Sea, Groepler and Kümmel (1988) observed for the first time intratunical bacteria. These ascidians usually settle on the claw-like holdfasts of *Laminaria* spp. Their translucent test forms the colony wall and enwraps the abdomina of the zooids (Fig. 1). The tunic tissue is composed of different cells, including conspicuous bladder cells which are embedded in a structureless matrix

(Groepler 1994). Fertilized eggs are released from ovarium to tunic, the embryonic development proceeds inside an envelope (Groepler 2002). In the initial state comma-shaped, fully developed embryos display tadpole-like forms with a rounded trunk enwrapped by a long tail. During embryonic development the tunic is generated.

This is a first report on differently shaped bacterial tenants in the tunic of adults and embryonic stages of the ascidian *D. migrans*. The regular occurrence of high bacterial numbers of the same morphotypes in the tunic matrix seems to be a general phenomenon here. The molecular identification and elucidation of the ecological role of the intratunical bacteria is in progress.

Materials and methods

Microscopic preparation

Samples

Fresh colonies of *D. migrans* were collected by the divers' group of Biologische Anstalt Helgoland (BAH) from the waters around Helgoland. Animals were kept for approximately 4 d in Petri dishes supplied with natural seawater, renewed daily.

Light microscopy

For inspection, whole, intact, small and relaxed colonies were transferred to microscopic slides. After reducing the water from samples, the animals were carefully squeezed between coverslip and slide until the tunic became sufficiently translucent for light microscopic investigation.

Scanning electron microscopy (SEM)

Preparation steps: (1) Colonies were fixed for 2 h in a buffer containing 2.5% glutaraldehyde, 0.1 M cacodylate and 0.45 M sucrose. (2) Samples were carefully rinsed with filter-sterilized distilled water (d.w.) and transferred in a drop of d.w. onto microscopic slides. (3) The tunic matrix was opened with a fine needle, causing the release of some intratunical bacteria. (4) Tissues of animals were removed, a coverslip put on and bacteria adhering to it were air-dried at ambient temperature. (5) Finally, the coverslip with bacteria was rinsed, dried, and stored until SEM inspection.

Transmission electron microscopy (TEM)

Glutaraldehyde-fixed samples were additionally treated in a buffer solution containing 1% OsO_4 for 1.5 h and stored until use.

Results

Intratunical bacterial morphotypes

The tunic of *D. migrans* usually harbors large numbers of different cell types (Groepler 1994). These cells make a thorough microbiological investigation inside the tunic difficult. A procedure which increases the pressure on the ascidian colonies is helpful in this respect. This is simply

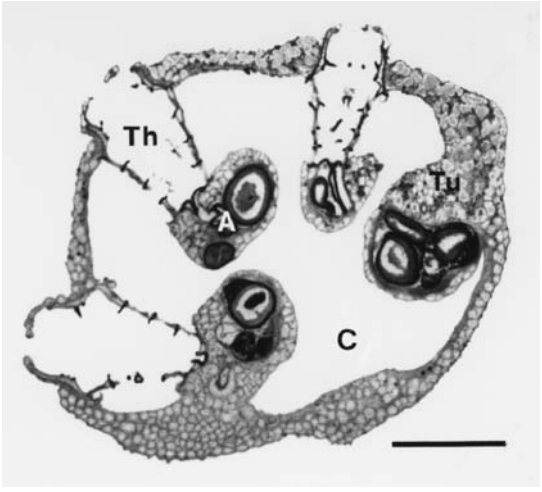

Fig. 1 Small colony of *D. migrans*; three zooids in longitudinal section; fixation after Bouin, staining with Alcian Blue/Nuclear Fast Red; *A* abdomen, *C* cloacal cavity, *Th* thorax, *Tu* tunic. *Bar* 0.5 mm

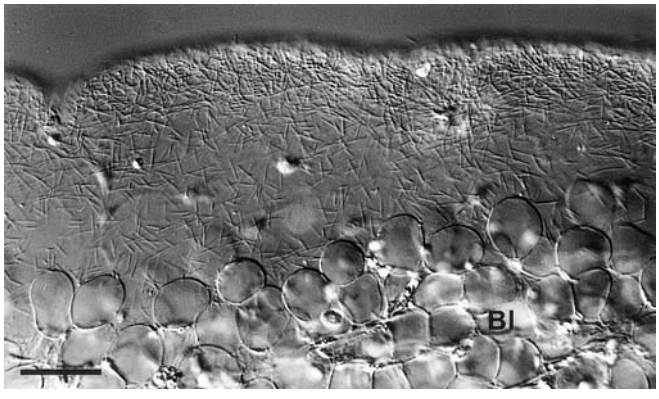

Fig. 2 Margin of tunic, top view. Squeezing a colony generated an elevation of tunic cuticula from bladder cells, *Bl*. The fluid-containing space harbors extremely high numbers of needle-like bacterial rods. *Bar* 50 µm

achieved by squeezing samples between coverslip and slides. Tunic bubbles are formed which are almost free of interfering tunic cells. Yet, the bubbles contain enormous quantities of intratunical bacteria (Fig. 2). Increasing pressure during evaporation of the samples frequently results in tunic burst, followed by a massive release of bacteria.

Light microscopic studies of natural intact tunics always reveal dense bacterial populations. The predominant group is represented by needle-like, long rods of 10–30 µm length and a width of about 0.5 µm (Fig. 3). The bacteria are motile by means of bipolar monotrichous flagella (Fig. 4), displaying swift sigmoidal movement. Forward direction can be instantly reversed. Forward and backward velocity seem to be the same. Dried samples occasionally show constrictions all over the bacterial surface; segments between the constrictions are well-rounded (Fig. 5). Segmentation has been not observed in

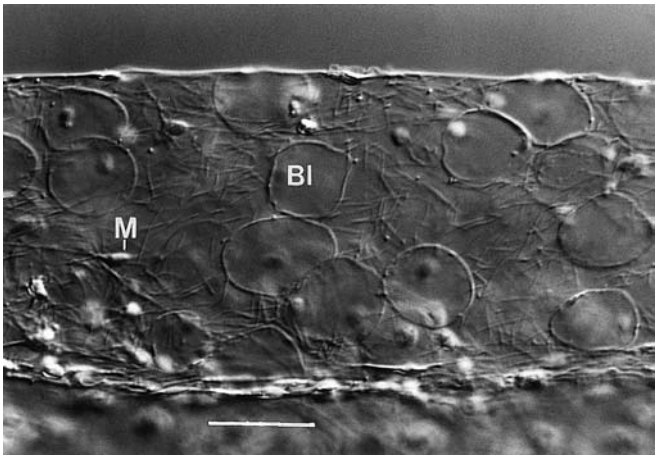

Fig. 3 Needle-shaped bacteria inside the tunic. Optical cross section. Matrix between cells colonized by bacteria. *Bl* bladder cell, *M* myocyte. *Bar* 50 μm

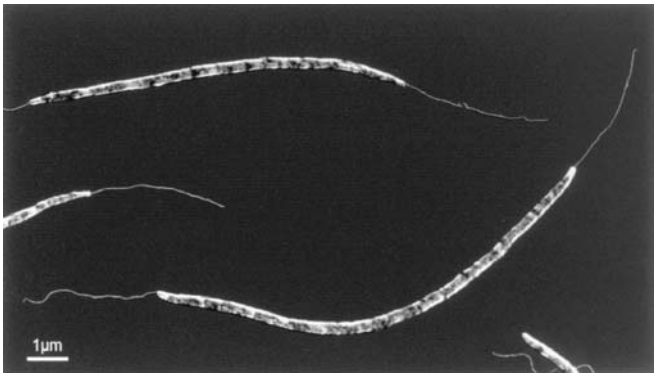

Fig. 4 Needle-like bacterial rods with bipolar monotrichous flagella. SEM micrograph. *Bar* 1 μm

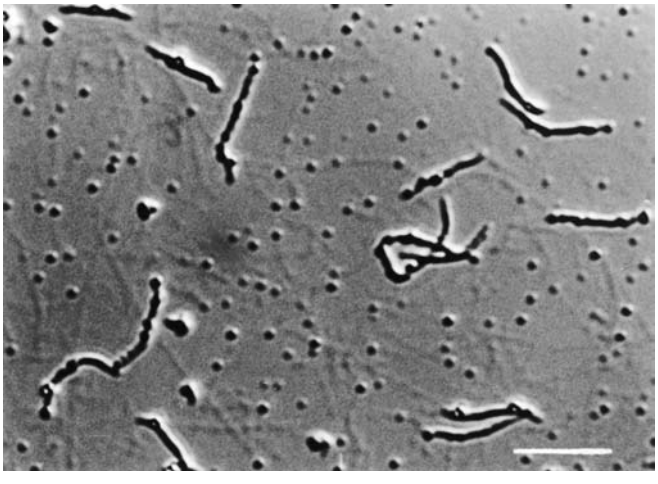

Fig. 5 Dried bacteria from tunic fluid. Needle-like bacteria with constrictions. The segments between the constrictions are well-rounded and show a similar shape as the masses of coccoid forms. *Bar* 10 μm

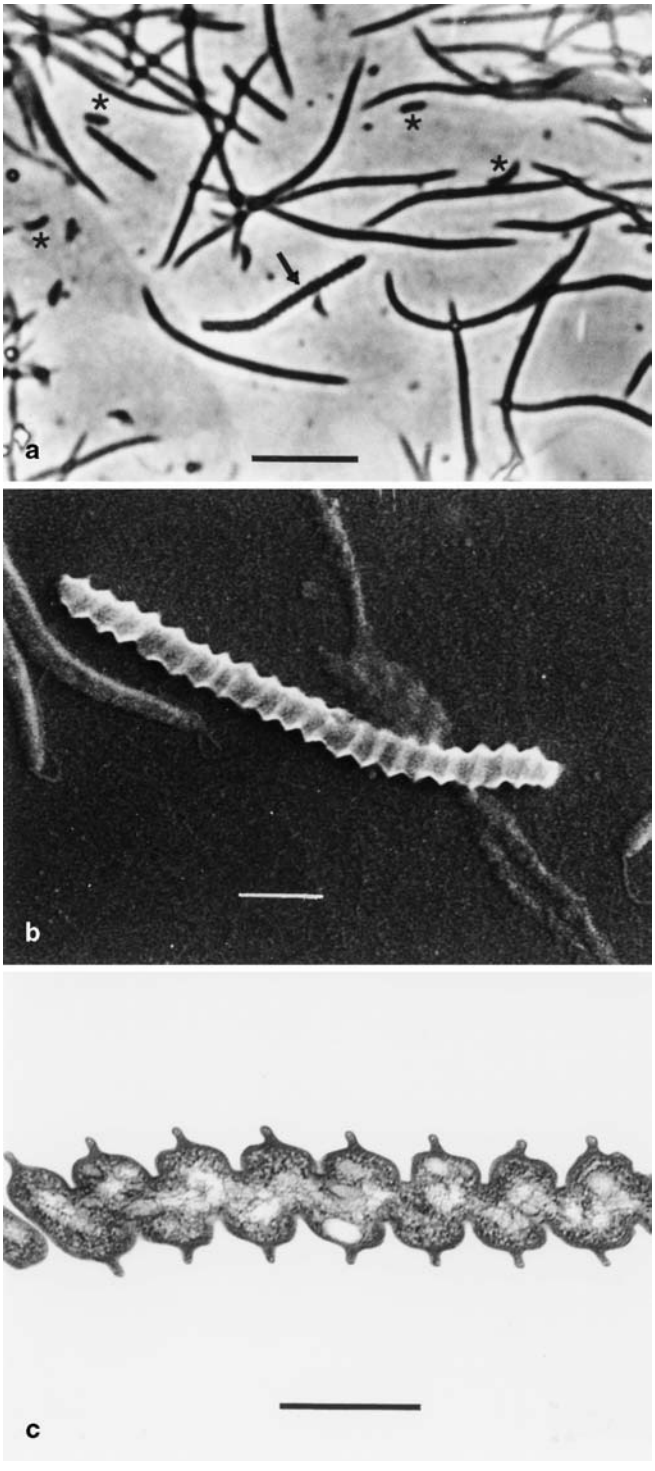

Fig. 6a–c Screw-shaped bacteria. **a** Different morphotypes from tunic fluid, fixed with glutaraldehyde. Besides many long (flagellated) rods, some short forms are visible (*asterisks*). *Arrow* indicates corkscrew shape. *Bar* 5 μm. **b** SEM micrograph. *Bar* 1 μm. **c** Sagittal section, TEM micrograph. Visible fold on vertex of screw structure. *Bar* 0.5 μm

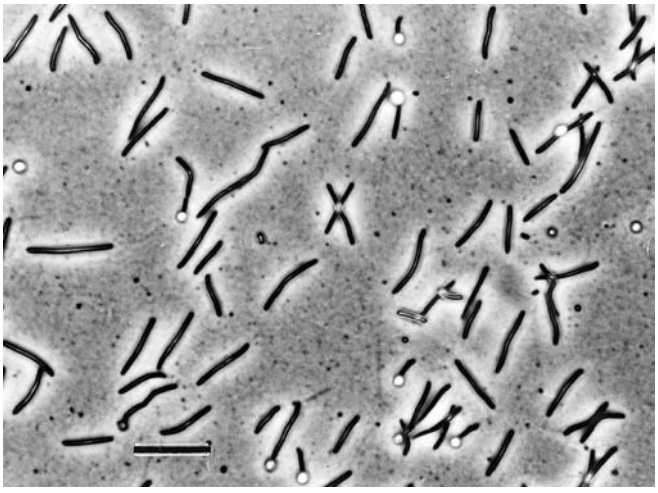

Fig. 7 Dried bacteria from tunic fluid. Several rods with a one-sided globule; diameters of globules are similar to isolated coccoid forms. *Bar* 10 μm

fresh samples. The tunic is also inhabited by another bacterial morphotype exhibiting an odd helical shape (length 4–10 μm, width about 0.5 mm) and blunt ends. SEM micrographs unveil their morphological structure, showing a single narrow surface fold of about 0.05 μm height (see Fig. 6a–c). These bacteria are relatively rare. Because unambiguous identification was only achieved with dried material, it is unknown whether these organisms are motile or not.

Apart from these bacterial groups, other bacteria of unknown significance were observed: (1) Short rods of 1–3 μm length and a width of 0.5 μm are quite abundant (Fig. 6a). Their width is similar to the needle-like bacteria. (2) Motile rods of different length with a ball-shaped globule at one end (Fig. 7). These organisms were not detected in each of the colonies tested. The diameter is comparable to the needle-like bacteria. (3) Small, immotile coccoid forms of 1 μm in diameter of varying quantities from low to high numbers (Fig. 5) are also found.

Development of bacterial community during embryonic development

As already described, embryonic development takes place inside the common test of the colony. Bacterial rods, detected by light microscopy, are observed for the first time in comma-shaped embryos. At this stage, bacteria are located in low numbers in the interspace between the epidermis of the embryo and the egg envelope (Fig. 8a). At later embryonic stages, bacteria were detected in higher numbers inside the newly developed tunic (Fig. 8b). All the samples of hatched larvae investigated so far have shown colonization of the test by needle-like bacteria.

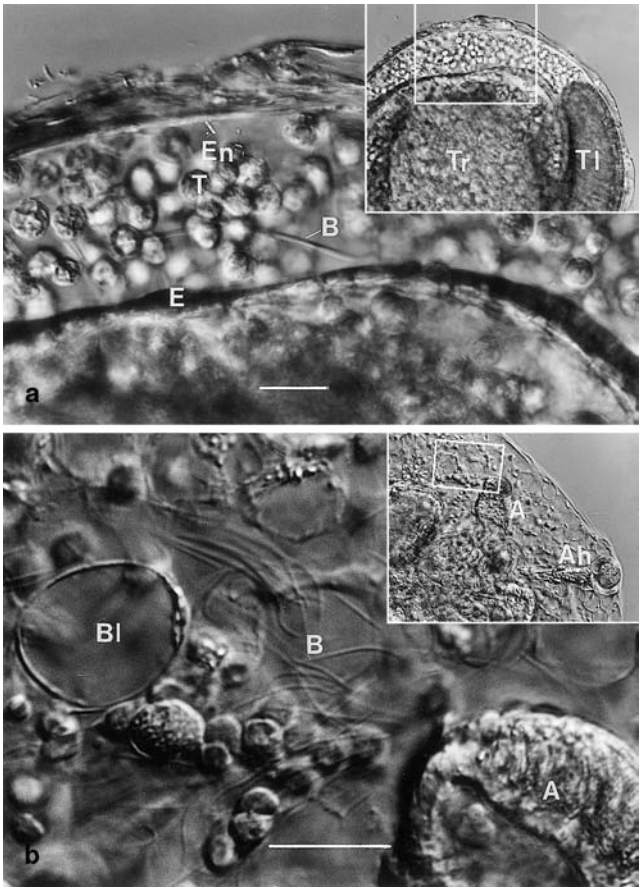

Fig. 8a, b Needle-like bacteria colonizing embryonic stages. **a** Comma-shaped embryonic stage; inserted micrograph. Enlarged display detail shows a few bacterial rods in the interspace between epidermis and egg envelope. *B* bacteria, *E* epidermis, *En* egg envelope, *T* test cells (?), *Tl* tail, *Tr* trunk. *Bar* 20 μm. **b** Late embryonic stage shortly before hatching; inserted micrograph. Enlarged display detail shows high numbers of needle-like rods in embryonic tunic. *A* ampulla, *Ah* adhesive papilla, *B* bacteria, *Bl* bladder cell. *Bar* 20 μm

Discussion

To our knowledge, needle-like bipolar flagellated bacteria are reported here for the first time as intratunical tenants of ascidians. In contrast there are reports on screw-shaped bacteria in the tunic of the tropical *Botrylloides simodensis* (Hirose and Saito 1992) and in *Clavelina miniata* (Hirose et al. 1996). Their size matches those of the bacteria found in ascidians from the North Sea. The screw-like morphology is also similar for both groups. The screw-shaped microorganisms of *D. migrans* seem to have no flagella. This is consistent with the description of the far-eastern organisms. However, gliding motility of these bacteria may be possible. A notable difference is a single surface fold of the bacteria from *D. migrans*. In contrast the screw-like bacteria of *Botrylloides simodensis* show three folds. Similarities in morphology and habitats do not allow the conclusion of close genetic relatedness.

Generally, there is no ecological and taxonomic information on these morphologically different bacteria.

Until now it has been unclear whether these bacterial tenants of *D. migrans* exclusively inhabit the tunic or whether they occur also as planktonic variants in the surrounding sea. Additionally, it is unclear by which mechanisms these bacteria enter the tunic of ascidian colonies. However, the tunic already harbors the above-described needle-like bacteria during the final embryonic stages before hatching. It can be assumed that these bacteria are transferred during sexual propagation from the parental colony to its offspring. This would make new infections from outside unnecessary. Indeed, needle-like rods were found in the interspace between the epidermis and egg envelope, suggesting that bacteria were transferred from parental tunic to interspace. It is conceivable that bacterial interspace colonization already occurs at an earlier stage of embryonic development, probably via spores. The colonization mechanism for the needle-like rods is still unresolved. This also applies for the globule-possessing short rods, coccoid forms, and the corkscrew-shaped bacteria. Light-microscopic tools reach their limits of detection here. Molecular approaches are in progress. Since the intratunical procaryotes show no evidence of rejection or degeneration, the associations between ascidian hosts and procaryotic cells seem to constitute a stable community. This favors the idea that the tunic of *D. migrans* provides a suitable environment for these intratunical bacteria.

At present there are more open questions than answers. A future task will be the taxonomic identification of the intratunical microbes, possibly using a molecular approach. Other projects would include attempts to cultivate intratunical bacteria. Further research efforts are needed to clarify the ecological role of the bacteria. In this context it is still unclear whether these bacteria are parasitic, symbiotic, or commensalic. For the elucidation of intratunical propagation modes, pure cultures are required.

Acknowledgements We are grateful to P. Pfundstein for the SEM and to R. Müller for the REM micrographs (University of Karlsruhe). Many thanks are due to the divers' group of BAH who reliably provided fresh ascidian material.

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