

Symbiotic bacteria in the nudibranch mollusk *Dendrodoris nigra*: fatty acid composition and ultrastructure analysis

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Abstract In the present study, the hypothesis that marine nudibranch mollusks harbor symbiotic bacteria was tested using analyses of fatty acids as biochemical markers and transmission electron microscopy of the tissues of *Dendrodoris nigra* (Gastropoda/Opisthobranchia/Nudibranchia). An aberrant level of the odd-numbered carbon chain and branched fatty acids, *iso*- and *anteiso*- that are specific for bacteria, was detected in the nudibranch tissues. Their amounts in the notum exceeded significantly that in the viscera. Rod-shaped gram-negative bacteria were revealed in the epithelial cells of the notum and the mantle edge as well as in the adjoining glycocalyx. These bacteria were enclosed in secondary vacuoles in the epithelial cells. The consequent stages of inoculation of the bacteria into the cytoplasm of epithelial cells, from adhesion to the apical surface to invagination of the cell membrane and formation of the vacuole with an enclosed bacterium, were observed. The presence of dividing bacteria suggests that the epithelium includes a renewable, dividing population of symbiotic bacteria. No bacteria were detected in the gonads and the digestive system. Probable functions of these symbiotic bacteria such as involvement in protection or defense from predators and environmental impacts as well as their nutritional role in the nudibranch are discussed.

Introduction

Dendrodoris nigra (Stimpson 1855) is a common Indo-West Pacific nudibranch species (Brodie et al. 1997). It occurs at moderately protected to moderately exposed rocky sites, where it is usually found under rocks during the day or crawling in the open at night, from the low intertidal to 5-m depth. *Dendrodoris* species, like many other nudibranch mollusks, feed on sponges. *Dendrodoris nigra* has been reported to feed on *Halichondria dura* and *Suberites* sp. (McDonald and Nybakken 1999).

Biology of nudibranchs and their ecology, particularly their food preferences, are insufficiently explored. Our knowledge of their lipid biochemistry is scarce (Martinez-Pita et al. 2005; Zhukova 2007). Moreover, analysis of the fatty acid composition of marine invertebrates could be valuable in determining their trophic habits and food sources (Dalsgaard et al. 2003). Thus, for example, the lipid composition of the tropical nudibranchs *Chromodoris* sp. and *Phyllidia varicosa* includes a number of very long-chain fatty acids called demospongiac acids in reference to their predation on sponges. Another unique feature of these sea slugs is the high abundance of various odd-numbered carbon chain and branched fatty acids (OBFA), predominantly *iso*- and *anteiso*-, specific for bacteria and usually called bacterial fatty acids (Zhukova 2007). It is intriguing because they are normally minor metabolites in most animals. There are apparently two alternative sources of OBFA in the nudibranchs. First, since the nudibranchs feed on sponges rich in bacteria, it is reasonable to suppose that these fatty acids may originate from bacteria associated with sponges, which are effectively transferred to the mollusk tissues, the mollusk being incapable of producing them. Second, based on the abundance of specific bacterial fatty acids, we hypothesized

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the presence of symbiotic bacteria in the nudibranch tissues.

Bacteria are known to produce various specific “bacterial-type” fatty acids, and a fatty acid analysis can be a good indicator of specific microorganisms, since different groups of bacteria have different fatty acid compositions (Perry et al. 1979; Gillan and Johns 1986). Fatty acids have been used to characterize symbiotic associations between bacteria and some marine invertebrates, such as bivalve mollusks, from a shallow-water hydrothermal vents (Conway and Cappuzzo 1991; Zhukova et al. 1992), deep-sea hydrothermal vent bivalves (Allen et al. 2001), amphipods (Pond et al. 1997), tubeworms (Pond et al. 2002), and gastropods (Pranal et al. 1996; Saito and Hashimoto 2010). Analysis of the fatty acid composition of marine invertebrates can serve as a valuable screening tool for detecting symbionts in species under study. The presence of high levels of specific bacterial fatty acids in the lipids of marine invertebrates has been shown to indicate an apparent bacterial symbiosis (Conway and Cappuzzo 1991; Zhukova et al. 1992; Rieley et al. 1999; Goffredi et al. 2005).

Symbiotic associations between microbes and marine invertebrates are widely distributed in nature; they increase biological and ecological diversity and enhance the evolutionary potential of different taxa (Saffo 1992; Dubilier et al. 2008; Chaston and Goodrich-Blair 2010). Many marine mollusks, typical representatives of Gastropoda, Bivalvia, and Cephalopoda, bear symbiotic microbes, but nudibranch mollusks are not presently known to be associated with symbiotic endobacteria. There is one report of symbiotic bacteria in the vestibular gland, associated with the female reproductive system, and in the egg masses of *D. nigra* (Klussmann-Kolb and Brodie 1999). There remains a question about the presence of intracellular symbiotic bacteria in the tissues of nudibranch mollusks.

Various techniques are available to investigate symbiotic microbes, including 16S ribosomal DNA sequence analysis, fluorescent in situ hybridization, transmission electron microscopy, stable isotopes, and fatty acid analysis. Symbiont-derived biochemical markers could serve as valuable screening tools for detecting symbionts in host tissues (Kharlamenko et al. 1995; McKenzie et al. 2000; Colaco et al. 2007). Fatty acid profiles of the animals may provide useful information on microbial groups with known and unequivocal lipid biomarkers, but fail to identify specific bacteria. Transmission electron microscopy (TEM) can reveal gram-negative and gram-positive bacteria, determine bacterium localization in the host cells and tissues, and suggest preliminary conclusions on the function of the symbionts. In light of these methodological advantages and limitations, combination of two or more techniques is preferable for investigating microbial symbiosis. For example, morphological and molecular

evidence has revealed a new symbiosis between a marine polychaete in the genus *Osedax* and members of the bacterial group Oceanospirillales, known for their heterotrophic degradation of complex organic compounds. Stable isotope and fatty acid analyses confirmed the role of the endosymbionts in the nutrition of the worm (Goffredi et al. 2005). The molecular, morphological, and stable isotopic characteristics of bacterial symbionts have been used to describe symbioses in the gill tissues of two mussels, *Adipicola crypta* and *A. pacifica*, collected from whale-falls on the continental shelf in the northwest Pacific (Fujiwara et al. 2010).

In the present study, we have used analysis of the fatty acids as biochemical markers and transmission electron microscopy to investigate the nudibranch *D. nigra*. The goal was to determine the sources of bacterial fatty acids in the nudibranch and to determine whether symbiotic bacteria are present in the nudibranch tissues. To examine the probable dietary origin of OBFA, the fatty acid composition of the sponge *Halichondria* sp., which is the prey of this nudibranch species, was also examined. To test whether the nudibranch tissues harbor symbiotic bacteria and whether this presence is explained by high amounts of odd and branched fatty acids in the mollusk, we examined the mantle edge, notum, digestive gland, and gonads of the nudibranch by light and transmission electron microscopy (TEM).

Materials and methods

Site and samples

Specimens of *Dendrodoris nigra* and *Halichondria* sp. were collected from the intertidal zone in rocky pools in Seongsan Sunrise Peak Bay in eastern Jeju Island, South Korea (33°27'30"N, 126°56'06"E), in October 2008. All samples were collected within an area of 30 m². The substrates varied from coarse sand, stones, and hard rock to calcareous algae. The nudibranchs collected were immediately placed in seawater from the site and transported to the laboratory. Five specimens of *Dendrodoris nigra* were used for biochemical and ultrastructural analyses.

Lipid analysis

Viscera were dissected and separated from the notum. Tissue samples were crushed, and lipids were extracted by homogenization in chloroform/methanol (1:2, by vol) according to Bligh and Dyer (1959). Fatty acid methyl esters (FAME) were prepared by sequential treatment of the total lipids with 1 % sodium methylate/methanol and 5 % HCl/methanol (Carreau and Dubacq 1978) and

purified by preparative silica gel thin-layer chromatography (TLC) using benzene as a solvent. *N*-Acylpyrrolidide derivatives of fatty acids were prepared by direct treatment of the FAME with pyrrolidine/acetic acid (10:1, by vol) in a capped vial for 30 min at 100 °C followed by chloroform extraction from the acidified solution and purification by preparative TLC developed in chloroform/acetone (9:1, by vol).

The FAME were analyzed on a Shimadzu GC-21A gas chromatograph equipped with a flame ionization detector, using a fused silica capillary column (Supelcowax-10, 30 m × 0.25 mm i. d., Supelco, Bellefonte, Pennsylvania) at 210 °C. Helium was used as a carrier gas at a linear velocity of 30 cm s⁻¹ (split ratio was 1:30). Injector and detector temperatures were 250 °C. Fatty acids were identified by a comparison with standard mixtures and equivalent chain length values. Identification was confirmed by gas chromatography–mass spectrometry (GC–MS) of their methyl esters and *N*-acylpyrrolidide derivatives using a model Shimadzu GCMS-QP5050A (Shimadzu, Kyoto, Japan) fitted with a MDN-5S capillary column (30 m × 0.25 mm i. d. Supelco, Bellefonte, Pennsylvania). Ionization of the samples was performed by an electron impact at 70 eV. The column temperature was programmed for 170 °C hold for 1 min, followed by an increase to 240 °C at a rate of 2° C min⁻¹, and then was held for 20 min. The temperature of the injector and detector was 250 °C. GC–MS of *N*-acylpyrrolidides was performed at a column temperature of 210 °C with 3 °C min⁻¹ increased to 270 °C held for 40 min. The GLSolution and GCMSSolution software (Shimadzu) were used for GC and GC–MS analyses. All data are the mean values of fatty acid analyses of five samples.

Microscopy

For transmission electron microscopy (TEM), samples of the mantle edge, notum, digestive gland, and gonads were fixed with 2 % glutaraldehyde in 0.1 M cacodylate buffer pH 7.2. After buffer rinses, tissues were post-fixed with 1 % osmium tetroxide in the cacodylate buffer pH 7.2, rinsed in distilled water and dehydrated in a graduated series of ethanol and isopropanol, and then embedded in Spurr's epoxy resin (Sigma, USA). Semi-thin sections (~1,000 nm) were prepared on a Leica EM UC6 ultramicrotome, stained with 1 % Methylene blue, and analyzed with the microscope Axio Imager (Carl Zeiss, Germany). Ultra-thin sections (~50 nm) were prepared on a Leica EM UC6 ultramicrotome, post-stained with 0.5 % uranyl acetate and lead citrate according to Reynolds (1963), and viewed using a Libra 120 electron microscope (Carl Zeiss, Germany).

Statistical analysis

The differences in fatty acid contents between the nudibranch tissues were tested using a Student's *t* test. Differences were considered statistically significant at $P < 0.05$.

Results

Fatty acid analysis

Sixty fatty acids were identified in the lipids of *Dendrodoris nigra*. The predominant fatty acids in whole nudibranch samples, occurring in concentrations exceeding 6 % of the total, were 16:0 (7.7 %), 16:1n-9 (13.3 %), 18:0 (6.4 %), 18:2n-6 (12.6 %), 20:4n-6 (8.2 %), and 26:2Δ5,9 (8.4 %), accounting for 56.3 % of the total fatty acids. The content of n-3 polyunsaturated fatty acids (PUFA) that included 18:4n-3, 20:5n-3, 22:6n-3, and 22:5n-3 (in total 5.9 %) was lower than that of n-6 PUFA (24.2 %). The nudibranch tissues were also characterized by a diversity of very long-chain fatty acids (VLCFA) identified as 25:2Δ5,9, 26:1 Δ9, 26:3Δ5,9,19, 27:2Δ5,9, 28:2Δ5,9, and the major 26:2Δ5,9 (8.4 %). This nudibranch also exhibited an abundance of various specific bacterial-type fatty acids, most of them corresponding to saturated and monounsaturated odd-numbered fatty acids with chain lengths C15 and C17. Various monomethyl branched fatty acids *iso*- and *anteiso*- with chain lengths from C14 to C19 were identified. Among them, particularly high levels of *iso*-15:0, *anteiso*-15:0, *iso*-16:0, *iso*-17:0, and *anteiso*-17:0 were found. In total, these OBFA in the lipids of *D. nigra* represented 15.4 % of the total fatty acids.

Some differences in the fatty acid composition were found between tissues of *D. nigra* (Table 1). Proportions of the different groups of fatty acids, such as Σ branched and odd fatty acids, Σ VLCFA, and Σ n-3PUFA, were clearly different between the notum and viscera (Fig. 1). Σ VLCFA, and Σ n-3PUFA, especially 20:5n-3 was significantly ($P < 0.05$) higher in viscera indicating the dietary origin of these fatty acids, whereas Σ OBFA was significantly higher ($P < 0.05$) in the notum than in viscera. Comparison of the sets of bacterial fatty acids of the notum and viscera revealed significant differences ($P < 0.05$) (Fig. 2). In particular, the high levels of *iso*-14:0, *iso*-15:0, *anteiso*-15:0, and 15:1 in the notum are noteworthy. The notum of *D. nigra* also contained slightly greater amounts of *iso*-16:0, *anteiso*-16:0, 17:1n-7, and 18:2n-6 as compared to the viscera. The acid 16:1n-9 was more abundant in the notum than in the viscera (19.7 % vs. 9.7 %).

The sponge *Halichondria* sp. was distinguished by a high proportion of VLCFA (Table 1; Fig. 1). Among them, the acid 26:2Δ5,9 (43.9 %) was the most abundant in the

Table 1 Fatty acid composition of *Dendrodoris nigra* and its prey sponge *Halichondria* sp. (% of total fatty acids)

| Fatty acids | <i>Dendrodoris nigra</i> | | <i>Halichondria</i> sp. |
|--------------|--------------------------|--------------|-------------------------|
| | Viscera | Notum | |
| iso-14:0 | 0.29 ± 0.01 | 0.96 ± 0.15 | – |
| 14:0 | 0.70 ± 0.26 | 0.46 ± 0.06 | 1.10 ± 0.14 |
| iso-15:1 | 0.24 ± 0.06 | 0.35 ± 0.06 | 0.10 ± 0.05 |
| iso-15:0 | 1.00 ± 0.02 | 2.52 ± 0.56 | 2.95 ± 0.35 |
| anteiso-15:0 | 0.46 ± 0.03 | 1.30 ± 0.12 | 0.20 ± 0.07 |
| 15:1 | 0.32 ± 0.07 | 0.90 ± 0.14 | – |
| 15:0 | 0.64 ± 0.09 | 1.09 ± 0.16 | 0.55 ± 0.12 |
| iso-16:0 | 1.13 ± 0.07 | 1.30 ± 0.19 | 0.70 ± 0.05 |
| anteiso-16:0 | 0.46 ± 0.09 | 0.62 ± 0.11 | 0.15 ± 0.07 |
| 16:1n-7 | 9.00 ± 0.3 | 16.55 ± 2.80 | 1.90 ± 0.28 |
| 16:0 | 5.95 ± 0.46 | 2.89 ± 0.52 | 5.40 ± 0.19 |
| 7Me-17:1n-11 | 0.83 ± 0.11 | 0.62 ± 0.13 | – |
| iso-17:0 | 1.39 ± 0.21 | 0.75 ± 0.09 | 1.30 ± 0.14 |
| anteiso-17:0 | 1.45 ± 0.22 | 1.01 ± 0.08 | 0.95 ± 0.09 |
| 17:1n-7 | 1.14 ± 0.73 | 2.56 ± 0.27 | – |
| 17:1n-11 | 0.12 ± 0.06 | 0.65 ± 0.37 | – |
| 17:0 | 2.44 ± 0.16 | 1.86 ± 0.25 | 0.30 ± 0.08 |
| 18:4n-3 | 0.41 ± 0.10 | 0.41 ± 0.08 | 0.25 ± 0.07 |
| 18:2n-6 | 10.20 ± 0.5 | 14.56 ± 0.75 | 1.05 ± 0.17 |
| 18:1n-9 | 5.54 ± 0.32 | 5.47 ± 0.45 | 2.45 ± 0.07 |
| 18:1n-7 | 5.42 ± 0.54 | 4.47 ± 0.30 | 3.95 ± 0.40 |
| 18:0 | 7.03 ± 0.66 | 5.12 ± 0.30 | 3.10 ± 0.19 |
| 19:1n-7 | 0.50 ± 0.11 | 0.29 ± 0.20 | – |
| 20:4n-6 | 8.87 ± 0.43 | 8.03 ± 1.41 | 4.40 ± 0.28 |
| 20:5n-3 | 4.95 ± 0.40 | 2.40 ± 0.3 | 3.20 ± 0.28 |
| 20:3n-9 | 0.94 ± 0.09 | 0.36 ± 0.10 | 0.30 ± 0.07 |
| 20:3n-6 | 1.26 ± 0.26 | 0.62 ± 0.27 | 0.30 ± 0.08 |
| 20:2 Δ5,11 | 1.13 ± 0.24 | 0.81 ± 0.16 | – |
| 20:2 Δ5,13 | 1.90 ± 0.7 | 1.40 ± 0.8 | – |
| 20:1n-11 | 1.62 ± 0.49 | 1.76 ± 0.54 | 0.95 ± 0.14 |
| 20:1n-9 | 1.08 ± 0.39 | 1.39 ± 0.49 | 0.20 ± 0.05 |
| 20:1n-7 | 0.89 ± 0.15 | 0.57 ± 0.10 | – |
| 22:6n-3 | 0.82 ± 0.12 | 0.26 ± 0.09 | 2.35 ± 0.21 |
| 22:4n-6 | 2.38 ± 0.31 | 2.90 ± 0.99 | 0.90 ± 0.08 |
| 22:5n-3 | 0.65 ± 0.12 | 0.37 ± 0.13 | 0.45 ± 0.11 |
| 22:5n-6 | 0.63 ± 0.1 | 0.60 ± 0.29 | – |
| 22:2 Δ7,13 | 1.00 ± 0.19 | 1.59 ± 0.62 | 0.25 ± 0.21 |
| 22:2 Δ7,15 | 0.98 ± 0.14 | 1.12 ± 0.47 | 0.35 ± 0.21 |
| 24:1n-7 | 0.06 ± 0.04 | – | 1.55 ± 0.17 |
| 25:2Δ5,9 | 0.38 ± 0.14 | 0.09 ± 0.05 | 1.15 ± 0.21 |
| 26:3Δ5,9,17 | – | – | 0.70 ± 0.28 |
| 26:3Δ5,9,19 | 0.08 ± 0.04 | 0.14 ± 0.05 | 1.30 ± 0.28 |
| 26:2Δ5,9 | 11.25 ± 1.12 | 6.14 ± 1.02 | 43.90 ± 3.39 |
| 26:1Δ19 | – | – | 0.70 ± 0.05 |
| 26:1Δ17 | – | – | 0.80 ± 0.04 |
| 27:2Δ5,9 | 0.20 ± 0.10 | 0.07 ± 0.06 | 2.70 ± 0.14 |

Mean ± SD (*n* = 5)

– non-detected. Other fatty acids include ai-14:0, 14:1, TMTD, 16:1n-7, br-17:1, 18:1n-5, i-19:0, 20:0, 21:5n-3, 21:2Δ7,13, 21:1n-9, 21:1n-7, 22:1n-11, 22:1n-9, 22:0, 23:2, 23:1n-11, 23:0, 24:2Δ5,9, 24:1n-9, 24:0, 26:2Δ5,9, 26:0, and 28:2Δ5,9 were present in trace amounts (≤0.2 %)

sponge as in its predator *D. nigra*. The amount of the OBFA was significantly lower ($P < 0.05$) in the sponge than in the viscera and notum of the nudibranch.

Microscopic analysis

Light and transmission electron microscopy on the mantle edge and the notum of the nudibranch was used to search for potential sources of the bacterial fatty acids found in its. The pseudo-stratified epithelium of the notum was quite similar to that of the mantle edge (Fig. 3). Different types of epithelial cells were observed in the mantle and notum: numerous ciliated epithelial cells and two types of specialized secretory glandular cells, with transparent and dense vacuoles (Fig. 3a). Ciliated cells were the most abundant cells in the epithelium. The apical surface of the epithelium was coated with a protective mucus layer

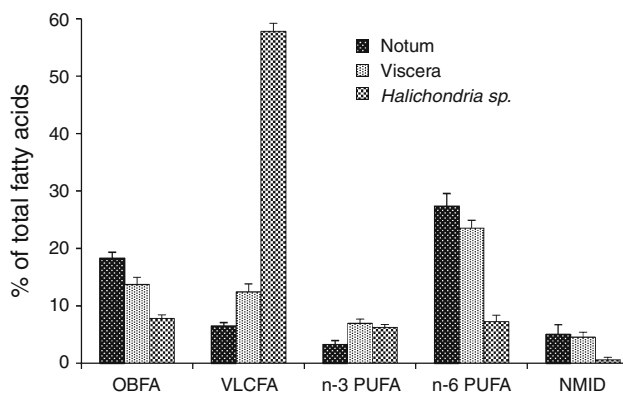
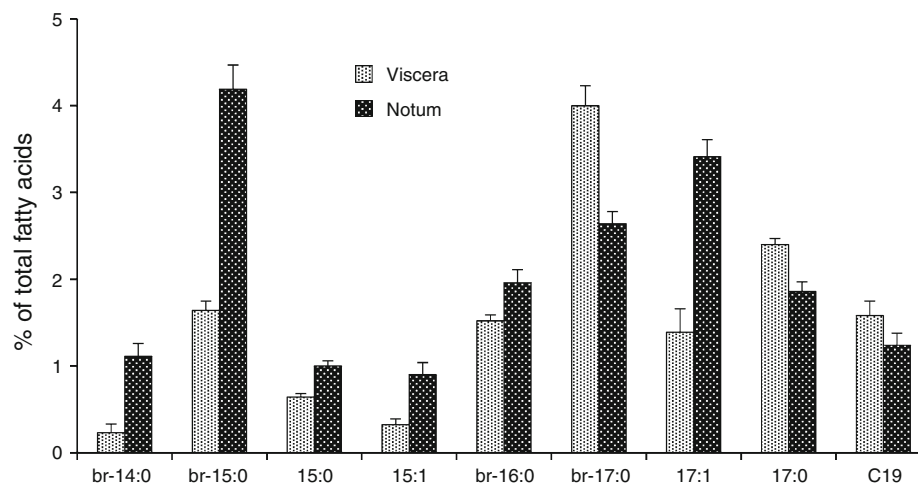


Fig. 1 Distribution of marker fatty acids in tissues of the nudibranch mollusk *Dendrodoris nigra* and its potential prey sponge *Halichondria* sp. (% of total fatty acids). Mean \pm SD ($n = 5$). OBFA odd-numbered carbon chain and branched fatty acids typical for bacteria, VLCFA very long-chain fatty acids specific for sponges, PUFA polyunsaturated fatty acids, NMID non-methylene interrupted fatty acids. Difference in the content of marker fatty acids between notum and viscera is significant ($P < 0.05$)

Fig. 2 Distribution of odd-numbered carbon chain and branched fatty acids, *iso*- and *anteiso*-, typical for bacteria in tissues of *Dendrodoris nigra* (% of total fatty acids). Mean \pm SD ($n = 5$). Difference in the content of these fatty acids between notum and viscera is significant ($P < 0.05$)



(glycocalyx); the basal part was surrounded by a thin wavy basal membrane, separating the epithelial layer from the underlying connective tissue (Fig. 3b). Epithelial cells were cylindrical, elongated, with a brush border and cilia. The apical membrane formed coated pinocytotic vesicles. The nuclei occupied a midbasal position, while the apical pole was vacuolated. The vacuolated apical pole of the cytoplasm space was occupied by a number of pinocytotic vesicles, multivesicular bodies, secondary lysosomes, and residual bodies. Near the nucleus, dictyosomes of the Golgi apparatus, cisternae of rough endoplasmic reticulum, vesicles of smooth endoplasmic reticulum, and mitochondria were observed.

Along with the epithelial cells, two types of glandular secretory cells were found. The first were large goblet cells with low electron density secretory vacuoles, and the second were cells containing electron-dense granules. Secretions from the glandular cells formed a protective polysaccharide layer (or glycocalyx) on the epithelial surface (Fig. 3a). Besides epithelial cells, there were migratory (wandering) cells, hemocytes, known to serve a defensive function through synthesis of humoral defense factors.

Transmission electron microscopy of the mantle edge and the notum of *D. nigra* revealed pleomorphic rod-shaped bacteria in the apical zone of the epithelial cells (Fig. 4a). The bacterial dimensions were $0.5 \times 1.5 \mu\text{m}$. Bacteria were enclosed in secondary vacuoles, as a rule one, and rarely two, bacteria per vacuole. We found from 1 to 4 bacteria in an epithelial cell. The bacteria were coated with two membrane layers separated by a thin periplasmic layer, typical of gram-negative bacteria (Fig. 4b). The surfaces of some bacteria were covered with thin fibrillae. Cytoplasm of the bacteria was uniformly granular; the nucleoid zone was strongly pronounced. We observed electron-transparent inclusions in the cytoplasm of some bacteria (Fig. 4c). A majority of bacteria were intact;

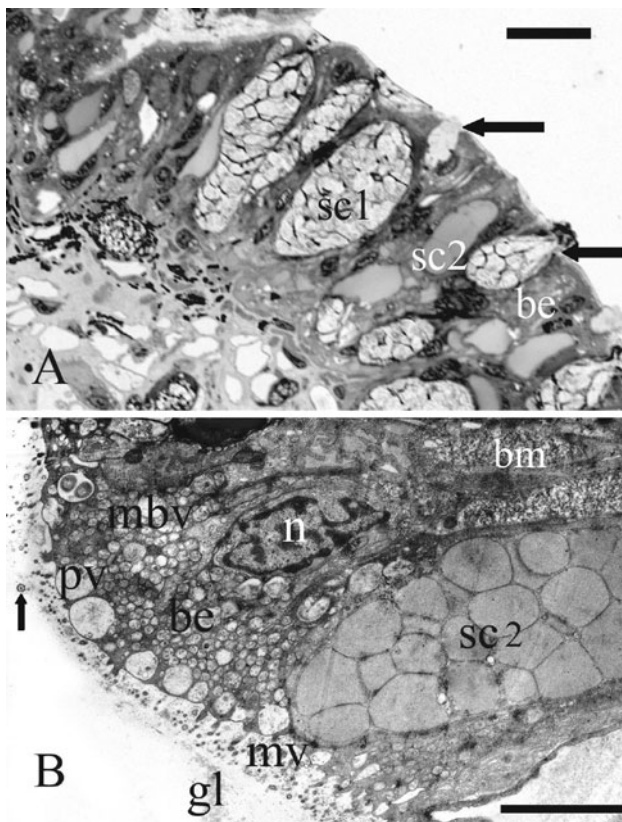


Fig. 3 *Dendrodoris nigra* notum epithelium. **a** Semi-thin section of notum stained with methylene blue shows three main types of cells in epithelium: epithelial cells (*be*) and two types of specialized secretory glandular cells with transparent (*sc1*) and dense vacuoles (*sc2*). Secretory glandular cells produce components of glycocalix (arrows). Scale bar 5 μ m. **b** Transmission electron micrograph of notum epithelium shows numerous pinocytotic vesicles (*pv*) and multivesicular bodies (*mbv*) in apical parts of epithelial cells and demonstrates process of nutrient absorption and intracellular digestion. Ciliated epithelial cells have microvilli (*mv*) and cilia (arrow); basal part is surrounded by a basal membrane (*bm*). Apical surface of epithelium is coated with glycocalix (*gl*). Scale bar 3 μ m

lysing bacteria were always single. This shows their resistance to lysosomal enzymes in epithelial cells and supports the hypothesis that these bacterial cells are endosymbionts. The presence of dividing bacterial cells also supports this proposition (Fig. 4d).

In addition to bacteria localized in the cytoplasm of epithelial cells of the notum and mantle, an extracellular subset of similar rod-shaped bacteria was observed in the glycocalix near the outer membrane of the epithelial cells (Fig. 5a). Morphologically, these are typical gram-negative bacteria with two layers in the outer membrane, which were often more wavy than those of intercellular bacteria (Fig. 5b). Their cytoplasm was electron-dense, with a distinct nucleotide zone. There was a trend toward increasing bacterial abundance in the apical membrane of the epithelial cells. We observed what appeared to be sequential stages of inoculation of bacteria into the cytoplasm of

epithelial cells—from adhesion to the apical surface to invagination of the cell membrane and formation of a vacuole with an enclosed bacterium (Fig. 5c, d). Among the extracellular bacteria in the glycocalix, we observed both intact and lysing bacteria; their cytoplasm content was released into the surrounding mucus layer (Fig. 5e, f). Bacterial debris was absorbed by the epithelial cells during pinocytosis. No bacteria were detected either in the gonads or in the digestive system.

Discussion

In general, the fatty acid composition of *Dendrodoris nigra* revealed a wide variety of fatty acids: saturated, mono- and polyunsaturated, straight, branched (*iso*- and *anteiso*-), even and odd, short-chain and very long-chain fatty acids. The aim of the study was not to undertake a comprehensive overall lipid analysis but rather to make an attempt to discern whether symbiotic bacteria were present in the host. A fatty acid analysis is useful in detecting bacterial signals that may indicate contributions by symbiotic chemotrophic bacteria to the nutrition of the host organism (Zhukova et al. 1992; Kharlamenko et al. 1995; Pond et al. 2002) and the presence of symbiotic heterotrophic bacteria (McKenzie et al. 2000; Goffredi et al. 2005).

A unique feature of the fatty acid composition of *D. nigra* was the unusually high abundance of the odd-chain (C13, C15, C17, and C19) and branched (*iso*- and *anteiso*-) fatty acids, specific for bacteria and usually called bacterial fatty acids (Fig. 1). Branched-chain fatty acids, in particular *iso*- and *anteiso*- forms of the odd-chain length 15:0 and 17:0 acids, are generally considered unique components of bacteria (Kaneda 1991). These fatty acids are often found in areas of high bacterial activity (e.g., Canuel and Martens 1993) and have been used as specific markers of bacteria in sediments (Perry et al. 1979). Odd-numbered fatty acids are mostly frequent in bacteria and lower plants or animals (Rezanka and Sigler 2009). They are normal as trace components in most marine invertebrates. Elevated relative concentrations of these compounds have been found in some deposit-feeding marine invertebrates (Meziane and Tsuchiya 2000; Bachok et al. 2003).

Bacteria are not always the only source of odd-chain fatty acids in invertebrates. The pteropod *Clione limacina* from Arctic and Antarctic waters was found to be rich in odd-chain fatty acids, dominant among which was 17:1n-8 reaching 18 % of the total, followed by 15:0 and 17:0, whereas odd-chain fatty acids were not detected in its only prey *Limacina helicina*, indicating that the atypical fatty acids were not ingested by *C. limacina* with the prey (Kattner et al. 1998). The authors suggest that de novo biosynthesis of odd-chain fatty acids occurs in the pteropod

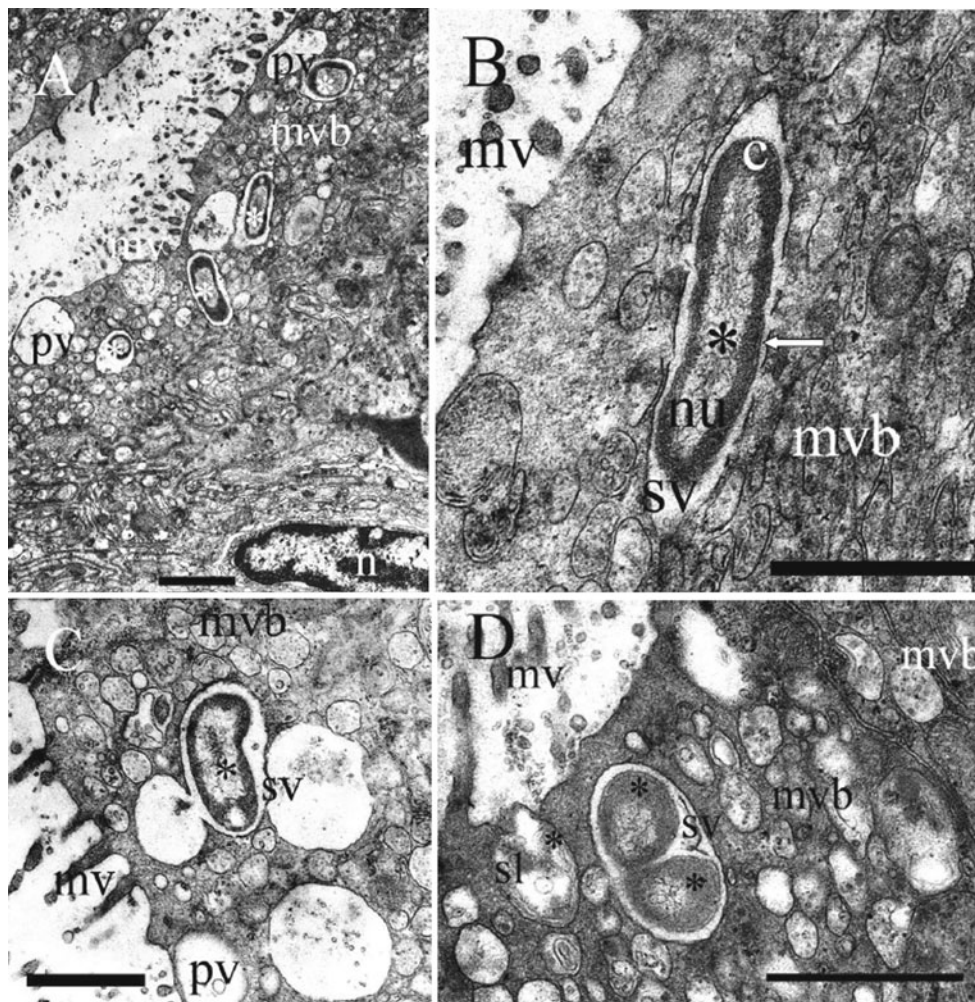


Fig. 4 Transmission electron micrographs of symbiotic bacteria within notum epithelial cells of *Dendrodoris nigra*. **a** Bacteria (asterisks) are localized in epithelial cells above nucleus (n). **b** A rod-shaped bacterium (asterisk) enclosed in secondary vacuole (sv) has dense cytoplasm and clearly marked area of nucleoid. Bacterium has twin membranes separated by a thin periplasmic layer (arrow) typical of gram-negative bacteria. **c** Bacteria (asterisk) with electron-

transparent inclusions containing nutrients. **d** Division of bacterium in secondary vacuole (sv) supports number of bacteria in epithelium. Degenerated bacteria (asterisk) are rarely apparent in secondary lysosome (sl) of epithelium. Microvilli (mv), multivesicular bodies (mvb), and pinocytic vesicle (pv) are present in images. Asterisks indicate bacteria. Scale bar 1 μ m

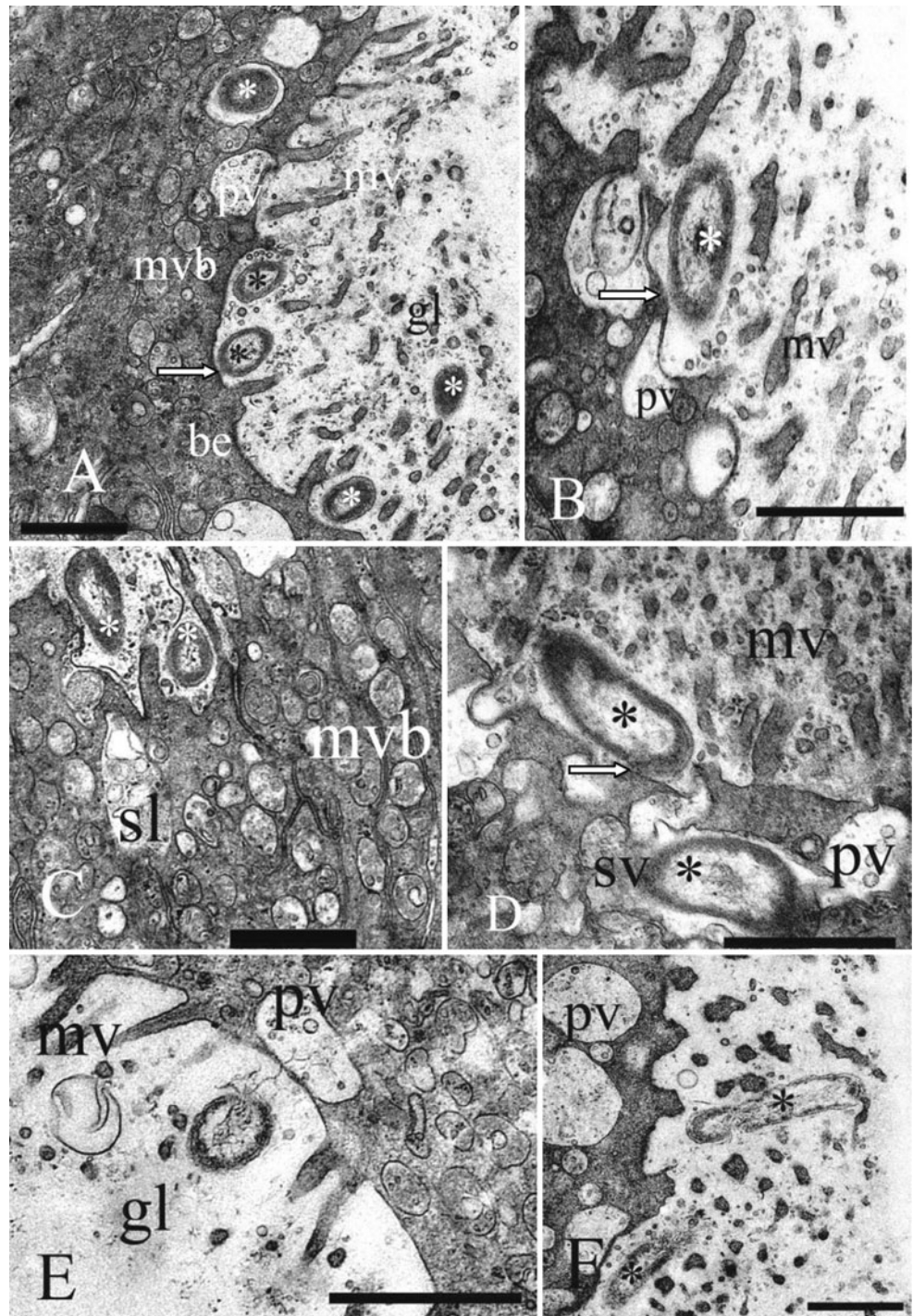
and initiates with propionate originating from dimethyl-beta-propiothetin, which is accumulated by *L. helicina* via phytoplankton uptake.

Comparison of the fatty acid composition of *D. nigra* and its prey sponge *Halichondria* sp. allowed us to elucidate the dietary source of OBFA in the nudibranch tissues. A high biological specificity of fatty acids, along with their structural diversity, permits to use fatty acids as biochemical markers for determining trophic relationships among species (e.g., Dalsgaard et al. 2003). Various odd and branched fatty acids are reported in many species of sponges (e.g., Dembitsky et al. 2003; Rezanka and Sigler 2009), including the *Halichondria* species, which are considered to be a food source for *D. nigra* (McDonald and Nybakken 1999). However, the specimens of *Halichondria*

sp. collected from the same habitat with *D. nigra* did not exhibit an abundance of OBFA, the amounts being significantly lower in the sponge than in the notum and viscera of the nudibranch. The sponge *H. panacea* from the Sea of Japan also contains low levels of straight and branched C15, C16, C17, C19 saturated and monounsaturated fatty acids; the concentrations of these fatty acids range from 0.1 to 0.6 % of the total fatty acids (Rodkina et al. 2003). Therefore, first, the proportions of the odd and branched fatty acids in the lipids of *D. nigra* were so high that it seems unlikely that bacteria from sponges are their only source for the sea slug. Moreover, the concentration of the total bacterial fatty acids in the notum significantly exceeded that in the viscera. This does not support the assumption that the OBFA result from the ingestion of

Fig. 5 Transmission electron micrographs of symbiotic bacteria in glycocalyx layer of *Dendrodoris nigra* notum epithelium and consequent stages of inoculation of bacteria into epithelial cells.

a Extracellular subset of gram-negative bacteria (*asterisk*) in glycocalyx (*gl*) of notum epithelium. **b** First stage of bacterium inoculation into an epithelial cell is accompanied by adhesion of bacterium to apical surface of cell. **c** Invagination of cell membrane and formation of a vacuole surrounding bacterium. **d** Different phases of secondary vacuole (*sv*) formation: adhesion of bacterium to membrane and invagination of secondary vacuole into cytoplasm of epithelial cell. **e** Lysis of bacterium and formation of pinocytotic vacuole (*pv*) containing bacterial debris. **f** Destruction of bacteria in glycocalyx layer. Microvilli (*mv*), multivesicular bodies (*mvb*), nucleus (*n*), and secondary lysosome (*sl*) are present in images. *Arrows* point to place of bacterial adhesion to membrane of epithelial cells. *Asterisks* indicate bacteria. *Scale bars* 1 μm



external bacteria associated with the sponges. OBFA, in contrast to the polyunsaturated fatty acids, are not essential for growth and reproduction of the mollusks, so it seems highly unlikely that OBFA from the food are accumulated preferentially in the nudibranch tissues. The mean percentages of bacterial markers (OBFA and 18:1n-7) associated with detritus are enhanced in the digestive gland of

filter feeding bivalves as compared to their other tissues (Galap et al. 1999; Silina and Zhukova 2007).

Second, the marked differences in a set of OBFA of the notum and viscera of *D. nigra* were obvious (Fig. 2). Comparison of the fatty acid composition of the notum and viscera exhibited different bacterial signals in these tissues. Concentrations of *iso*-14:0, *iso*-15:0, *anteiso*-15:0,

iso-16:0, 15:0, 15:1, and 17:1 acids in the notum were elevated, whereas in the viscera, the acids *iso*-17:0, *ante*-*iso*-17:0, and 17:0 were dominant. Taking into consideration the specificity of the fatty acid composition of different species of bacteria, we suggest that distinct bacterial microflora supply the nudibranch with OBFA. Similarly, based on the marked differences in fatty acid composition between microflora associated with whale bones and the polychaete *Osedax* sp. inhabiting the whale-bone and hosting symbiotic bacteria, Goffredi et al. (2005) suggest that the worm obtains the bacterial fatty acids from the symbionts rather than from free-living microbes.

Thus, it seems unlikely that OBFA found in *D. nigra* originate exclusively from the consumed sponges. The origin of these compounds from symbiotic bacteria, at least partly, seems plausible. The presence of considerable amounts of a specific bacterial fatty acid (*cis*-vaccenic) previously allowed us to show that the bivalve *Axinopsida orbiculata* has symbiotic chemoautotrophic bacteria. This bivalve is among the invertebrates from a shallow-water hydrothermal community in the Kraternaya Bight (Kharlamenko et al. 1995). In a similar way, the occurrence of appreciable quantities of 16:1n-7 and 18:1n-7 in some species of brittlestars in comparison with non-symbiotic brittlestar species represents a contribution from subcuticular heterotrophic bacteria to the hosts (McKenzie et al. 2000). We therefore suggest that a high relative abundance of bacterially derived odd-numbered carbon chain and branched fatty acids may indicate the presence of heterotrophic bacteria in the nudibranch tissues.

Indeed, TEM showed the presence of rod-shaped bacteria in the cytoplasm of epithelial cells and in the glycocalyx layer covering the epithelium of the notum and the mantle of *D. nigra*. The bacteria found appeared to be gram-negative based on the structural peculiarities of their cell wall, which had twin membranes separated by a thin periplasmic layer. Symbiotic bacteria possessed the fimbriae, which are known to provide adhesion of bacteria to the apical membrane of epitheliocytes (Duguid et al. 1976; Clegg and Gerlach 1987) facilitating colonization of epithelial cells of the nudibranch host by symbiotic bacteria. Our ultrastructural observations show that the bacteria penetrate into the cytoplasm of epithelial cells through phagocytosis, accompanied by invagination of the outer cell membrane of epitheliocytes and formation of a vacuole with an enclosed bacterium. The data indicate that these bacteria are facultative as opposed to obligate symbionts. The bacteria localized in the cytoplasm are acquired at the expense of recruitment of extracellular bacteria from seawater. Horizontal endosymbiont acquisition from the environment to the host by means of a phagocytosis-like mechanism involving special “pit-like” structures on the apical cell membrane is reported for the hydrothermal vent bivalve

Bathymodiolus azoricus (Kadar et al. 2005) and the obligate tubeworm (Siboglinidae, Polychaeta) endosymbiosis (Nussbaumer et al. 2006). Acquisition of symbiotic bacteria from the environment by host squid and fishes (i.e., horizontally) is common in bioluminescent symbioses (e.g., Dunlap et al. 2009). The level of colonization varies during the life cycle of the host and depends on environmental conditions, for example, nutrient availability and oxygen concentration (Johnson and LePennec 1995; Bates 2007). We suggest that the symbiotic bacteria in *D. nigra*, similarly to different bacterial pathogens and endosymbiotic bacteria, take advantage of host cell autophagy for successful colonization of the host cells (Campoy and Colombo 2009). The larvae of hydrothermal vent tubeworms (Vestimentifera, Siboglinidae) are symbiont-free, and the bacterial symbionts colonize the developing tubes of the settled larvae entering the hosts through the skin. Each generation of tubeworms must be newly colonized with its specific symbiont (Nussbaumer et al. 2006). Thus, the symbiont transmission process provides mechanisms common to both pathogenic infections and beneficial host–symbiont interactions. Although the details of transmission and symbiont selection vary among associations between microorganisms and animals, comparisons of diverse mutualistic associations reveal a number of common features, including restriction of symbiont diversity during transmission, and glycan–lectin interactions during partner selection and recruitment (Chaston and Goodrich-Blair 2010).

Bacteria in the cytoplasm of epithelial cells of the notum and mantle of *D. nigra* are enclosed in a secondary vacuole. The distinct membrane separating the bacteria from the host cytoplasm may play a role in regulating metabolite transfer between the host and the symbionts, and/or in protecting the bacteria from host enzymes (Piel 2004). Symbiotic bacteria revealed in the nudibranch acquire nutrition from the host. This is confirmed by the TEM images (Fig. 4c), which show fusion of multivesicular bodies, which are involved in intracellular digestion, with the membrane of the secondary vacuole enclosing the bacteria. A similar process was described in the review by Fader and Colombo (2009). No signs of lysis were observed; this indicates that the bacteria associated with the nudibranch epithelium are resistant to phagocytosis. The nudibranch epithelial cells were unable to consume bacteria, in contrast to brittlestars, where symbionts are phagocytosed and lysed by their epidermal cells (McKenzie and Kelly 1994).

The presence of dividing bacteria suggests that the epithelium of the notum and the mantle edge of the nudibranch *D. nigra* include a renewable, self-producing population of symbiotic bacteria. Evidently, the life cycle of symbiotic bacteria includes a sequence of stages both inside the epithelial cells (in the apical part) and outside of

these cells—in the glycocalyx coating the surface of the epithelium. The bacteria in the glycocalyx sometimes undergo destructive lysis, with their components being utilized by the epithelial cells in the course of intracellular digestion. The high concentration of typical bacterial fatty acids in the lipids of the nudibranch agrees well with the results of TEM and confirms that the lysed bacterial cells are utilized by the mollusk tissues.

The nudibranch symbiosis differs markedly from other marine symbioses based on autotrophy. A majority of symbiotic bacteria are chemoautotrophic. A fundamental feature of many symbiotic microorganisms is that they provide the host with nutrients that would otherwise enhance their own growth and proliferation rates (Saffo 1992; Dubilier et al. 2008; Chaston and Goodrich-Blair 2010). Other symbiotic functions for marine microbes include nutrition through direct uptake of dissolved organic matter in seawater (Wilkinson et al. 1984), production of secondary metabolites (e.g., Schmidt and Donia 2010), and detoxification of toxic compounds (Stewart and Cavanaugh 2006). It has been suggested that symbionts may participate in the synthesis of compounds involved in cellulose digestion in wood-boring bivalves (Xu and Distel 2004). Also, bacteria associated with cultured bivalve larvae have been shown to produce antibiotics (Fdhila et al. 2003). Some squids maintain a population of symbiotic bacteria of the genus *Vibrio* and use the bioluminescence of these bacteria at night in an antipredatory behavior (e.g., Jones and Nishiguchi 2004). Previously, symbiotic bacteria were found in the mucus surrounding the egg capsules as well as, between and partly aligned with the microvilli, in the vestibular gland of *D. nigra*. It is assumed that these bacteria may play a role in the breakdown of the mucous layers and the egg capsule during intracapsular development, thus ensuring the release of the veliger (Klussmann-Kolb and Brodie 1999).

There is increasing evidence in marine invertebrates that symbionts may play an important role in producing bioactive chemicals or their precursors within the host (e.g., Proksch et al. 2002; Schmidt and Donia 2010). Animal hosts, as well as embryos and developing larvae, are often afforded protection against fungi, pathogenic bacteria, and predators by compounds produced by bacterial symbionts (Lindquist 2002, 2005; Lopanik et al. 2006). These symbionts are sometimes the true producers of the host's secondary metabolites that have defensive and protective functions for their hosts. Many biologically active compounds including toxic and deterrent secretions have been isolated from nudibranchs in the family Doridaceae (Wägele et al. 2006), where defensive chemicals are often concentrated in the mantle (Avila and Durfort 1996). Since our biochemical and ultrastructural research revealed symbiotic bacteria only in the epithelial cells of

integumentary tissues, the notum and the mantle, we suspect that these bacteria might be involved in protection or defense from predators or environmental impacts. A nutritional role for these bacteria in the nudibranch *D. nigra* is also possible. A high level of bacterial fatty acids in the nudibranch may serve as evidence that the symbiotic bacteria provide the host with nutrients.

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