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Internal storage and production of symbiotic bacteria in the reproductive system of a tropical marine gastropod

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Abstract The vestibular gland and egg masses of *Dendrodoris nigra* (Gastropoda: Opisthobranchia: Nudibranchia) were investigated histologically and ultrastructurally. A detailed description of the ultrastructure of the gland and the egg masses is given, and the presence of symbiotic bacteria in the vestibular gland and identical-appearing bacteria in the egg masses is reported for the first time. Hypotheses about the function of these bacteria in the reproductive system of *D. nigra* are presented.

Introduction

Dendrodoris nigra (Stimpson, 1855) is a widespread Indo-West Pacific nudibranch mollusc of the family Dendrodorididae. The genus is notable because of its highly glandular anterior digestive system (Wägele et al. 1999) and lack of radula. The species is reported to feed on non-reticulated sponges of the genera *Halichondria* and *Tedania* (Young 1969; Brodie et al. 1995).

The presence or absence of a vestibular gland, associated with the female reproductive system, has been an important character for discrimination of *Dendrodoris* species (Brodie et al. 1997). To date nothing has been published about the function of this gland in opisthobranchs. Although a vestibular gland is described for several nudibranch families, i.e. Aegiridae, Dorididae, Dendrodorididae, Chromodorididae, Flabellinidae, such glands are not considered to be homologous between

these families (Gosliner 1994). This highlights the importance of comparative investigations of reproductive morphology in the study of molluscan phylogenetic relationships. While studying the functional anatomy and ultrastructure of *Dendrodoris nigra* a very unusual cellular structure was observed in the vestibular gland. On further investigation by transmission electron microscopy (TEM) this gland was shown to contain large numbers (several thousands) of densely packed, rod-shaped symbiotic bacteria.

Because of their density, appearance and orientation, we are certain that these bacteria are not external contaminants but are stored within the nudibranch for a functional purpose and are actively reproducing.

A general account of endosymbioses in invertebrates is given by Saffo (1992). Bacterial symbionts are reported for many different invertebrate groups, e.g. Insecta (Schilthuizen and Stouthammer 1997), Isopoda (Juilfs and Wägele 1987), Echiurida (Menon and Arp 1993), Monoplacophora (Haszprunar et al. 1995), Aplousobranchia (Scheltema et al. 1994), Gastropoda and Bivalvia (Waterbury et al. 1983; Stein 1984; Reid and Brand 1986; Trager and DeNiro 1990) and Cephalopoda (Bloodgood 1977; McFall-Ngai and Ruby 1991; Kaufman et al. 1998). The involvement of symbiotic bacteria in reproduction has not previously been reported for gastropods.

The histology of *Dendrodoris nigra*, including that of its vestibular gland, is reported in detail by Wägele et al. (1999). The present paper includes a further description of the histology of the gland, a description of the histology of the egg mass, as well as the first description of the ultrastructure of the vestibular gland and the egg masses of *D. nigra*. The possible functional role of the bacteria found residing in both of these components of the reproductive system is also discussed.

Materials and methods

In total four specimens of *Dendrodoris nigra* and four egg masses of the species, collected in the field as well as laid in captivity, were

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used for this study. The molluscs were collected intertidally and kept overnight in aquaria before being relaxed using methol flakes.

Two specimens from Dingo Beach (20°05'S; 146°49'E) found in July 1997 (sizes 48 and 53 mm extended crawling length) were preserved in 8% formalin in seawater for a minimum of 4 weeks. The same preservation method was applied to pieces of an egg mass found together with an adult specimen at Cockle Bay, Magnetic Island (19°11'S; 146°49'E) in August 1997, and two egg masses laid by additional individuals (not used further in this study) in captivity. The specimens and egg masses were examined histologically using the methacrylate technique (Kulzer, Technovit 7100), as utilized by Wägele (1998). Serial sections (2 to 3 µm thick) were stained with toluidine blue, and single sections of the vestibular gland and the digestive system were also Gram stained according to Twort (see Bandcroft and Stevens 1977).

Two specimens from Cockle Bay found in August 1997 and October 1997 (58 and 40 mm, respectively) were dissected for electron microscopy, and pieces of an egg mass laid in captivity by a different individual from Cockle Bay (not used further in this study) were preserved for TEM at different developmental stages (2, 4 and 8 d). The samples were preserved in 3% glutaraldehyde buffered in Millepore-filtered seawater. For post-fixation 1% osmium-tetroxide in seawater was used, and the samples were embedded in Spurr's resin. Ultra-thin sections were stained with uranyl acetate and lead citrate and studied using a Zeiss 109 electron microscope.

Results

Vestibular gland

The vestibular gland of *Dendrodoris nigra* is approximately 1 mm in size and visible, by dissection, within the body cavity close to the genital aperture. This gland opens via a small ciliated duct into the distal oviduct near the vestibulum. Internally the gland itself presents a characteristic convoluted appearance (Fig. 1A). The cells are cuboidal to prismatic and bordered by a very distinct, purple-staining apical fringe. This fringe consists of a dense layer of microvilli (Fig. 1B). In all specimens examined we found (between and partly aligned with the microvilli and also free within the lumen of the gland) many thousands (4 to 5 µm⁻²) of identical, densely packed, rod-shaped gram-negative bacteria (Fig. 1C, D), averaging 0.38 µm in diameter. The epithelial cells of the gland contain a centrally located nucleus with the nucleolus usually visible. Numerous vesicles of varying sizes from 0.5 to 3.0 µm are found in the apical part of the cell, their number decreasing basally. The contents of these vesicles are unknown. Mitochondria are spread throughout the cell. In the basal part of the cells a few Golgi complexes, the endoplasmic reticulum and a very few vesicles are also present. A thick layer of connective tissue containing numerous small vesicles with unknown contents is present close to the basal lamina of the cells.

Egg mass

The egg mass (Fig. 2A) is composed of distinct layers: on the outer edge a multi-layered mucus cover surrounds

the whole mass. Inside this cover is a wide, unstructured mucus matrix consisting of loose mucus fibres within which are embedded inner mucus layers. Embedded within these inner mucus layers are the egg capsules, each containing one embryo. Thus a thin inner mucus layer surrounds each capsule. Ultrastructural investigation of an egg mass showed that bacteria with an identical appearance to those observed in the vestibular gland were also present in the mucus surrounding the egg capsules. In the 2-d-old egg mass, the bacteria were located mainly in the outer mucus cover (Fig. 2B) and only relatively few (<2 µm⁻²) were present within the mucus matrix. In the egg mass preserved after 4d, the overall structure of the egg mass had not changed, but the number of bacteria had increased (<5 µm⁻²) and they had spread throughout all the mucus layers. The bacteria had penetrated to the outside of the egg capsules. On the eighth day of development (shortly before hatching) many thousands of bacteria were present in the dissolving egg mass (5 to 10 µm⁻²). The majority of bacteria were located in the mucus matrix and around the egg capsule which, in comparison to the developmental stages described above, had become very thin (Fig. 2C). No bacteria could be detected within the digestive system of the larvae.

Discussion and conclusions

Because of the high density, consistency and number of bacteria, as well as their alignment between the microvilli of the epithelial cells of the vestibular gland, we are certain that these bacteria are not external contaminants but are actively reproducing and stored within the nudibranch for a functional purpose. Symbiotic bacteria have been documented in other marine invertebrates and shown to have various functional roles in the different taxa. They can be associated with the cuticle (Holland and Neilson 1978), epidermis (Haszprunar et al. 1995), or the integument (Menon and Arp 1993) playing a protective role in the latter by providing sulfide detoxification in a sulfide-rich environment. They can be a direct food source for the host (Stein 1984; Trager and DeNiro 1990) or facilitate consumption of otherwise unavailable energy sources (Juilfs and Wägele 1987; Distel and Roberts 1997). In squid endosymbiotic bacteria of the light organ affect bioluminescence (Ruby 1996). In some invertebrate groups symbiotic bacteria are also reported to be involved with reproduction. A similar relationship to that reported in this present paper, i.e. colonization of symbiotic bacteria in an accessory gland of the female reproductive system, has been reported for different species of squid (Bloodgood 1977; Lum-Kong and Hastings 1992; Kaufman et al. 1998). However, the function of these bacteria is unknown. Biggs and Epel (1991) reported that the bacteria of the accessory gland in squid are deposited on the egg capsule sheath in the egg mass. This is very similar to what we have found for the egg masses of *Dendrodoris nigra*.

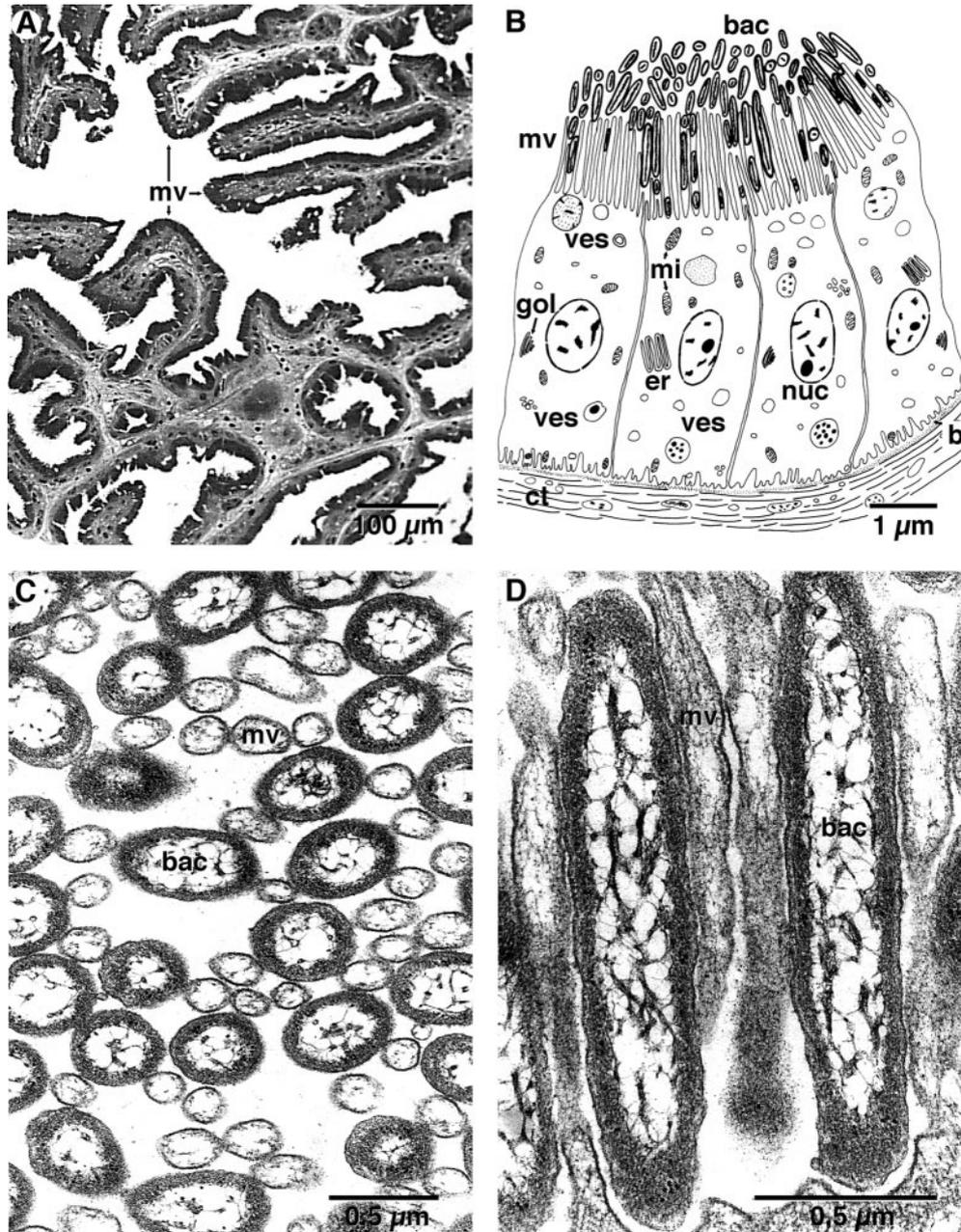


Fig. 1 *Dendrodoris nigra*. Vestibular gland: **A** histological cross-section of gland; **B** schematic outline of the ultrastructure of the gland; **C** TEM cross-section of bacteria between microvilli of vestibular gland; **D** TEM longitudinal section of bacteria between microvilli (*bac* bacteria; *bl* basal lamina; *ct* connective tissue; *er* endoplasmic reticulum; *gol* golgi vesicles; *mi* mitochondria; *mv* microvilli; *nuc* nucleus; *ves* vesicles)

The vestibular gland of *Dendrodoris nigra* and its bacteria are unique in that they are an integral and consistent part of the reproductive system, and such a relationship has not been reported before in the Gastropoda.

Since a histological and ultrastructural examination of the entire digestive system revealed no bacteria, we do not believe that our bacteria are playing a digestive role

in dendrodorid molluscs. The histological results of Wägele et al. (1999) confirm that a duct from the vestibular gland enters the oviduct just prior to its opening into the genital atrium. This position would seem to reflect the gland's function in reproduction, more specifically the final stages of egg mass production. Bacteria have also been found in the egg masses of the Antarctic nudibranch species *Austrodoris kerguelensis* and *Tritoniella belli* (Wägele 1989), although no vestibular glands are reported for these species.

Although the function of the vestibular bacteria is as yet unclear, discovery of apparently identical bacteria embedded within egg masses suggests 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 veligers. Other authors have

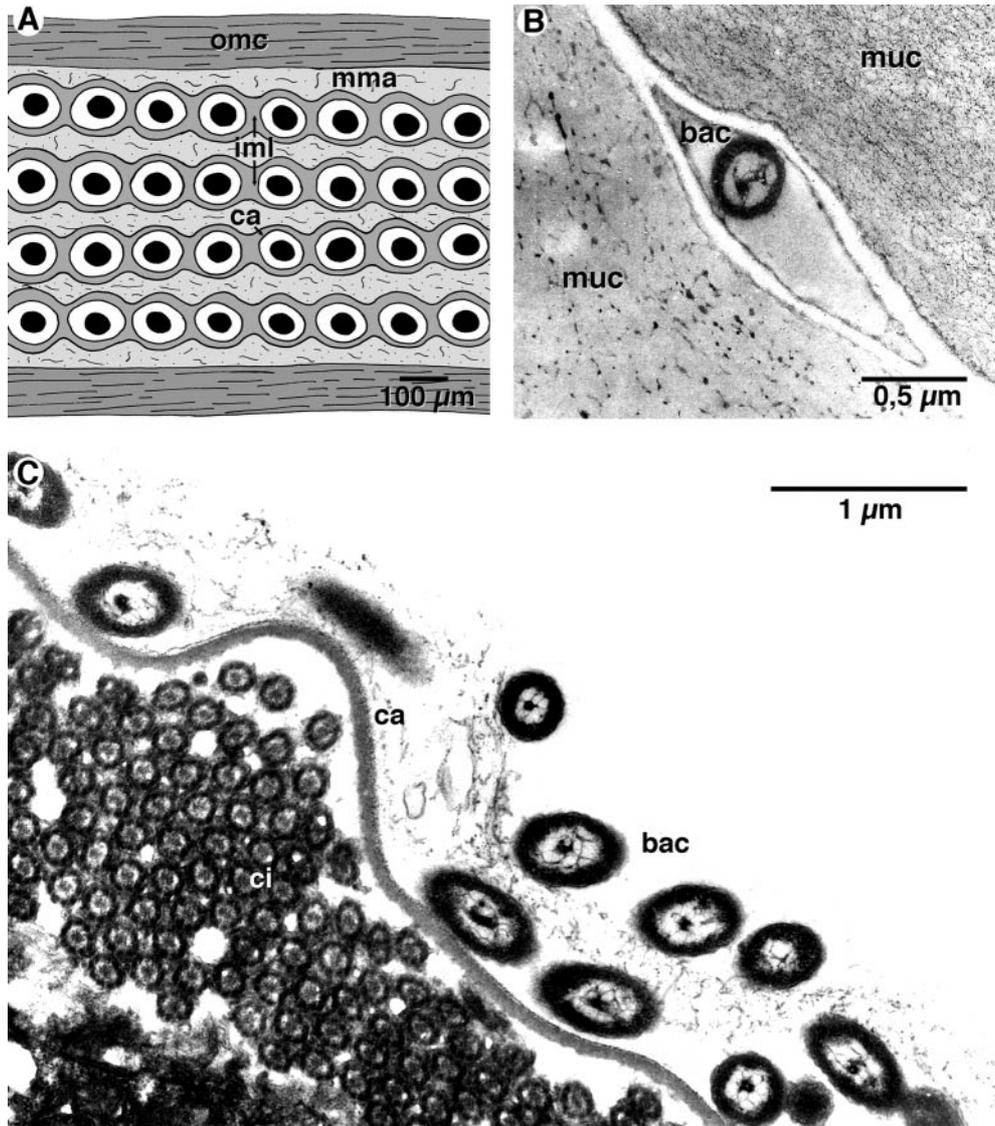


Fig. 2 *Dendrodoris nigra*. Egg mass: **A** schematic outline of a longitudinal section through a piece of egg mass; **B** single bacteria embedded in the outer mucus layer of a 2-d-old egg mass; **C** bacteria along the outer edge of the egg capsule in an 8-d-old egg mass (*bac* bacteria; *ca* capsule; *ci* cilia; *iml* inner mucus layer; *mma* mucus matrix; *muc* mucus; *omc* outer mucus cover)

previously suggested this to be the case (Eyster 1986; Carroll and Kempf 1990). Harris (1975) also stated that colonization by bacteria, protozoans and nematodes, as well as water movement, were important for the breakdown of egg masses. The majority of veligers in our cultivated egg mass hatched by Day 8. This is close to the expected intracapsular development period reported by Rose (1985) for this species, but could be influenced by strong aeration in our holding tanks. This level of aeration may also have influenced the rate of bacterial multiplication in our egg masses.

A protective role of the bacteria implanted in the egg mass for the embryos of *Dendrodoris nigra* is also possible. Gil-Turnes et al. (1989) and Gil-Turnes and

Fenical (1992) showed that bacteria protect embryos of shrimps and lobsters from fungal infection. Biggs and Epel (1991) also stated that bacteria associated with the egg sheath of the squid *Loligo opalescens* may defend the developing embryo against colonization by parthenogenic organisms.

It is also possible that the bacteria in the egg masses of *Dendrodoris nigra* are passed to the next generation by enclosing them in the egg masses, or that they may function as a food source for the larvae. Although this latter hypothesis is speculative, it is supported by reports from other molluscs (Stein 1984; Trager and DeNiro 1990).

Further studies are required to classify the bacteria and to investigate the role of such bacteria in the life history of *Dendrodoris nigra*. We also need to determine if vestibular glands found in other nudibranch species contain any such symbionts.

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