Cah. Biol. Mar. (2002) 43 : 321-324

New aspects of the symbiosis in the provannid snail *Ifremeria nautilei* from the North Fiji Back Arc Basin

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Introduction

The hydrothermal vent systems of the Western Pacific are dominated by flourishing populations of molluscs that harbor bacterial endosymbionts and can attain a major ecological role as primary consumers. In the back arc basins of Manus (MB), Lau (LB), and North Fiji (NFB) (Fig. 1), the provannid snail *Ifremeria nautilei* Bouchet & Warén, 1991, is one of the most abundant macrofaunal species. Unlike non-symbiotic provannids, *I. nautilei* is a large species that can reach shell lengths up to 8.5 cm (Desbruyères et al., 1994) and individual weights of >80 g (including the shell, calculated by unpublished regressions from our own NFB material). Population densities in the LB were estimated to reach 500-700 adults m^{-2} (Bouchet & Warén, 1991), which corresponds to an estimated biomass of >15 kg m^{-2} wet weight. Settling in the vicinity of hot hydrothermal emissions or within the moderately heated diffuse flow of hydrothermal fluids mixed with sea water, these gastropods are exposed to temperatures of 3-20 °C and to moderate to high concentrations of both sulphide and methane.

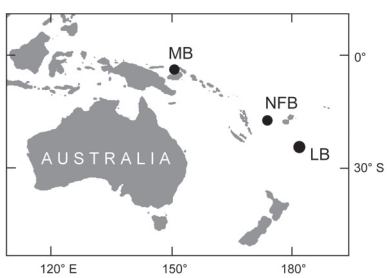

Figure 1. Geographical locations of the back arc basins in the western Pacific: MB = Manus Basin, NFB = North Fiji Basin, LB = Lau Basin.

While the symbiotic relationship between *I. nautilei* and its microbial endobionts was recognized soon after the discovery of this species, the nature of the symbiosis and thus, the trophic strategy of this snail is still disputed. Gal'chenko et al. (1993) examined material from the MB using electron microscopy and detected two types of procaryotic cells in the gill tissues that differed in size and internal structure. The smaller morphotype was similar in structure and appearance to previously described sulphide-oxidizing symbionts, while the larger morphotype contained stacked cytoplasmic membranes typically found in methane-oxidizing symbionts. Support for the presence of chemoautotrophy came from enzymatic assays with cell-free extracts of gill tissue, and showed the activity of ribulose-1,5-bisphosphate carboxylase (RubisCO) - a key enzyme for the autotrophic fixation of CO_2 in the Calvin-Benson cycle. The presence of methylotrophic bacteria was supported by the detection of an enzyme involved in the methylotrophic pathway, methanol dehydrogenase (MDH). Stable carbon isotopic ($\delta^{13}C$) ratios between -41‰ and -35 ‰ in various tissues of the host animals additionally supported the conclusion that *I. nautilei* from the MB derive their organic carbon in part from methanotrophic organisms (Gal'chenko et al., 1993).

The consistent occurrence of a dual symbiosis in *I. nautilei* was questioned by Windoffer & Giere (1997), who found only a single bacterial morphotype, typical of sulphide-oxidizing symbionts, in specimens from the NFB. Bacteria resembling methane oxidizers, i.e. with internal membranes, were never observed. The $\delta^{13}C$ ratios of the NFB *I. nautilei* (-33.7‰ to -31.4‰) were less negative than those of the MB *I. nautilei* and were within the range of values found in hydrothermal vent bivalves that derive their organic carbon exclusively from chemoautotrophic symbionts. Thus, the authors assumed that methanotrophic symbionts do not occur in *I. nautilei* from the NFB. These

results indicate variable patterns in the symbiosis of *I. nautilei*.

Windoffer & Giere (1997) concluded that the observed differences could be due to deviating evolutionary developments in the two host populations from MB and NFB, that are separated by some 3000 km. Molecular studies by Kojima et al. (2000) on MB and NFB *I. nautilei* showed that these two host populations are genetically distinct on the basis of their cytochrome oxidase I genes. Alternatively, the absence of methanotrophic symbionts in the examined NFB specimens could have resulted from potential within population variability, which may not have been detected because of insufficient sampling. A scenario in which the presence or absence of methanotrophic symbionts might be related to environmental factors, such as the concentration of methane in the local hydrothermal fluids, is also conceivable. To clarify this issue, we used ultrastructural and molecular methods to study the symbiosis in *I. nautilei* specimens collected in 1998 during the R/V "Sonne" expedition 134 in the LHOS hydrothermal vent field in the North Fiji spreading axis.

Material and methods

The LHOS hydrothermal vent field is located in 2000 m water depth and is characterized by diffuse emanations of sea water diluted hydrothermal fluids with temperatures of 5-20 °C, and concentrations of HS^- and methane up to 126 µmol l^{-1} and 36,700 nl l^{-1}, respectively (Halbach & Shipboard Scientific Party, 1998; Halbach et al., 1999). Animals were collected by a video controlled grab at several stations in the LHOS field separated from each other by some 100-300 m. The analyses of the material included morphological and genetic characterization of the symbionts, immunocytochemical localization of associated enzymes, and stable carbon analyses of host tissues. In addition to the SO 134 material from 1998, we also reexamined some specimens from the Windoffer & Giere (1997) study, which were collected in 1995, during the R/V "Sonne" expedition SO 99 in the same area.

Results and Discussion

Ultrastructural analyses of the gill tissues of the new 1998 *I. nautilei* material initially seemed to confirm earlier results from the 1995 expedition. The bacteriocytes in the gill tissues of all specimens were densely packed with rod-shaped bacteria of the sulphide-oxidizer morphotype, and a second morphotype was originally not observed. Amplification of the bacterial 16S rDNA gene and subsequent phylogenetic sequence analysis revealed the presence of a gamma proteobacterium that clustered with a group of known thiotrophic symbionts from other chemoautotrophic hosts. We verified the sequence by fluorescence in situ hybridization (FISH) with specifically developed oligonucleotide probes. The chemoautotrophic nature of the rod-shaped cells was confirmed by ultrastructural localization of RubisCO using an immunocytochemical in situ hybridization technique. In this method, the presence of RubisCO form I is visualized by transmission electron microscopy (TEM) after incubation of symbiont containing tissues with polyclonal antiserum specific to RubisCO form I (Krieger et al., 2000). The $\delta^{13}C$ ratios (–36.6‰ to -31.7‰) in the gills of the 1998 NFB material exhibited a wider range than measured previously in 1995 NFB animals, but were on average less negative than those of the *I. nautilei* specimens from MB and in the range of values found in hydrothermal vent bivalves that harbor only sulphide-oxidizing symbionts (Trask & Van Dover, 1999).

A second symbiont, which we had not seen in the previous TEM analyses, was finally discovered with FISH in the 1998 NFB *I. nautilei*. Hybridization with a general oligonucleotide probe specific to alpha proteobacteria resulted in fluorescence signals which displayed very regular and distinct distributional patterns. Based on preliminary FISH studies, the alpha proteobacterial symbiont occurred in most *I. nautilei* specimens, but in only low abundances within each individual and was limited to defined areas near the dorsal edges of the gill filaments (Fig. 2). Subsequent careful electron microscopical scrutiny

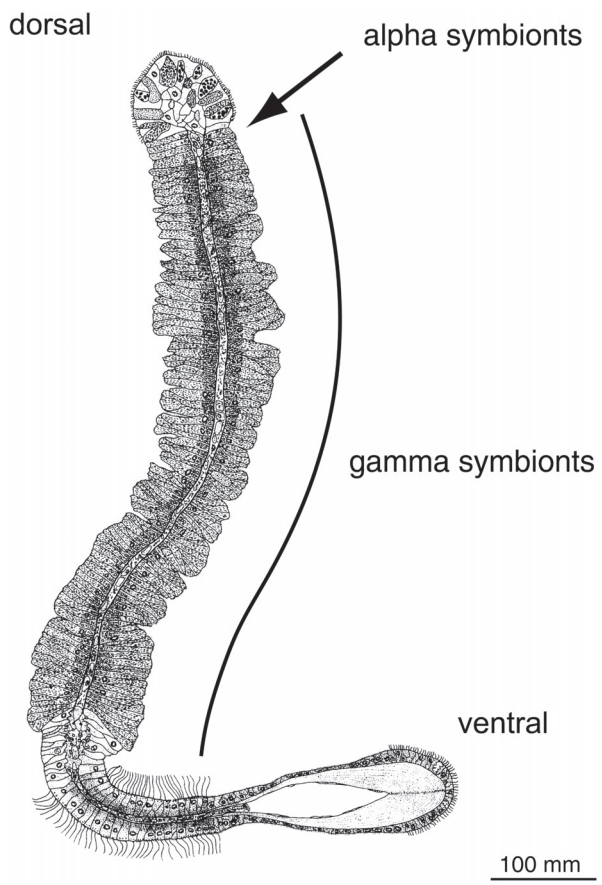

Figure 2. Schematic median cross section of an *I. nautilei* gill filament illustrating the distribution patterns of the symbionts (modified after Windoffer & Giere, 1997). The gamma proteobacteria are abundant in all bacteriocytes, while the alpha proteobacteria are spatially restricted to limited areas near the dorsal edge of the ctenidial filament, where they co-occur in low abundances with the gamma proteobacteria.

of *I. nautilei* specimens from both the 1995 and 1998 R/V "Sonne" expeditions revealed the existence of a second bacterial morphotype with internal membranes resembling those of type I methanotrophs (Fig. 3). The spatial distribution of this membrane-containing symbiont near the dorsal edges of the gill filaments corresponded to that of the alpha proteobacterial symbiont observed with FISH. The low abundances of the membrane-containing symbiont in *I. nautilei* from NFB is in contrast to *I. nautilei* from the MB, in which the methanotrophic morphotype has a considerable share in the total number of symbiotic cells (Beck & Sobjinski, 1999) and expresses measurable enzymatic activity of MDH (Gal'chenko et al., 1993).

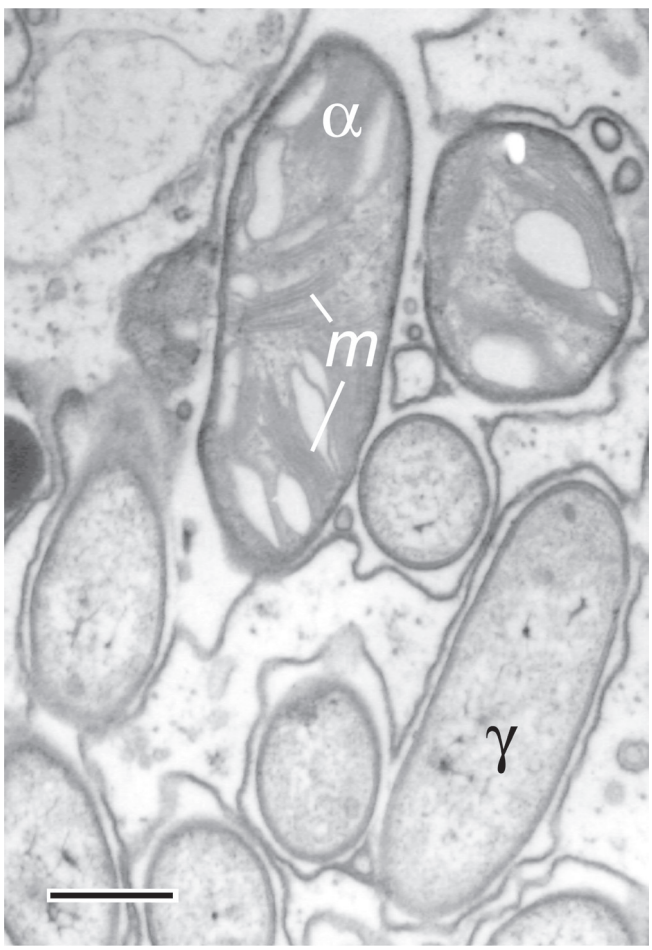

Figure 3. Cross section of the dorsal area of a gill filament where both morphotypes of symbiotic bacteria co-occur (TEM). γ = abundant chemoautotrophic morphotype, α = second morphotype with stacks of internal membranes (*m*) resembling those of type I methanotrophs. Scale 0.5 µm.

Conclusions

Ifremeria nautilei from the NFB harbors two types of bacterial symbionts in its gills. Sulphide-oxidizing gamma proteobacterial symbionts are abundant and distributed evenly in all bacteriocytes and all individuals. A second symbiotic morphotype with internal membranes was overlooked in earlier work because of its very low abundance and restricted spatial distribution. We are currently using electron microscopy in situ hybridization, comparative 16S rRNA analyses, and amplification of functional enzymes typical of methane-oxidizing bacteria, to confirm more definitively whether the membrane-containing symbionts in *I. nautilei* are alpha proteobacteria that can utilize methane. In addition, we are collaborating with Japanese colleagues (Hidetoshi Urakawa et al.) that are using molecular methods to investigate the symbiosis in *I. nautilei* from the MB. To this date, all known methanotrophic symbionts belong to the gamma proteobacteria. Thus, the *I. nautilei* membrane-containing symbiont may represent a novel methanotrophic symbiont phylotype.

Acknowledgements

We thank the captain and crews of R/V "Sonne" cruises SO 99 and SO 134. Many thanks to two anonymous reviewers who helpfully commented the manuscript. This work was supported by grants from the German Federal Ministry for Education, Research and Technology, within the HIFYFLUX II program under contract number 03G0134C, and the Max Planck Society.

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