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SHORT COMMUNICATION

Shift from widespread symbiont infection of host tissues to specific colonization of gills in juvenile deep-sea mussels

Cecilia Wentrup[1], Annelie Wendeberg[2], Julie Y Huang[3], Christian Borowski[1] and Nicole Dubilier[1]

[1]*Max Planck Institute for Marine Microbiology, Symbiosis Group, Bremen, Germany;* [2]*Department of Environmental Microbiology, UFZ, Helmholtz Centre for Environmental Research, Leipzig, Germany and* [3]*Department of Microbiology and Immunology, Stanford University, Stanford, CA, USA*

The deep-sea mussel *Bathymodiolus* harbors chemosynthetic bacteria in its gills that provide it with nutrition. Symbiont colonization is assumed to occur in early life stages by uptake from the environment, but little is known about this process. In this study, we used fluorescence in situ hybridization to examine symbiont distribution and the specificity of the infection process in juvenile *B. azoricus* and *B. puteoserpentis* (4–21 mm). In the smallest juveniles, we observed symbionts, but no other bacteria, in a wide range of epithelial tissues. This suggests that despite the widespread distribution of symbionts in many different juvenile organs, the infection process is highly specific and limited to the symbiotic bacteria. Juveniles ⩾9 mm only had symbionts in their gills, indicating an ontogenetic shift in symbiont colonization from indiscriminate infection of almost all epithelia in early life stages to spatially restricted colonization of gills in later developmental stages.

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Introduction

Associations with chemosynthetic bacteria have evolved independently in at least four lineages of marine bivalves (Dubilier *et al*., 2008). In all these associations, the symbionts are generally restricted to the gills in adult bivalves (Taylor and Glover, 2010), except hosts that transfer their symbionts directly to their offspring and harbor symbionts in their gonads (Endow and Ohta, 1990; Cary and Giovannoni, 1993). In adult *Bathymodiolus* mussels, symbionts have only been observed in the gills (Distel *et al*., 1994; Duperron, 2010). These mussels occur worldwide at hydrothermal vents and cold seeps and harbor their intracellular sulfur- and methane-oxidizing symbionts in gill cells called bacteriocytes. *Bathymodiolus* is assumed to acquire its symbionts from the environment, but the developmental stage at which this occurs is not known (Won *et al*., 2003; Duperron *et al*., 2007). Post-larval *Bathymodiolus azoricus* and *B. heckerae* as small as 0.12 mm appear to already harbor symbionts in their gills based on transmission electron microscopy (TEM) observations of bacteria morphologically similar to the sulfur- and methane-oxidizing symbionts (Salerno *et al*., 2005). These bacterial morphotypes were also found in the mantle epithelia of post-larval and juvenile *B. azoricus* and *B. heckerae* (0.12–8.4 mm). Similarly, in *B. childressi* juveniles (4–8 mm) TEM revealed bacteria that looked like the methane-oxidizing symbionts in gills as well as mantle and foot epithelia, and labeling experiments showed that these bacteria fixed carbon from methane (Streams *et al*., 1997). These studies suggest that *Bathymodiolus* is colonized by its symbionts at a very early stage and that other organs besides the gills also contain symbionts.

In this study, we focused on the following questions: (1) Do the symbionts indiscriminately infect all host tissues of juvenile *Bathymodiolus*? (2) Are the symbionts the only bacteria that colonize juvenile mussels? (3) Given that adult mussels are assumed to only have symbionts in their gills, is there a developmental stage at which the broad

Correspondence: C Wentrup or N Dubilier, Max Planck Institute for Marine Microbiology, Symbiosis Group, Celsiusstr. 1, Bremen 28359, Germany.
E-mail: cwentrup@mpi-bremen.de or ndubilie@mpi-bremen.de
or A Wendeberg, Department of Environmental Microbiology, UFZ, Helmholtz Centre for Environmental Research, Permoserstraße 15, Leipzig 04318, Germany.
E-mail: annelie.wendeberg@ufz.de
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colonization of host tissues ends? To answer these questions, we analyzed eight *B. puteoserpentis* and five *B. azoricus* juveniles (4–21 mm) from two vent sites on the Mid-Atlantic Ridge (Supplementary Table S1) by fluorescence in situ hybridization (FISH). Semi-thin sections of whole juveniles were examined with symbiont-specific probes as well as a general eubacterial and a negative probe (see Supplement for more details about methods).

Results

Symbionts broadly colonize tissues of smallest juvenile mussels

We observed symbiont-specific FISH signals indicating the presence of both the sulfur- and the methane-oxidizing symbionts in gill bacteriocytes of all 13 *Bathymodiolus* juveniles (Figure 1). In the smallest individuals (4–7 mm), we also found

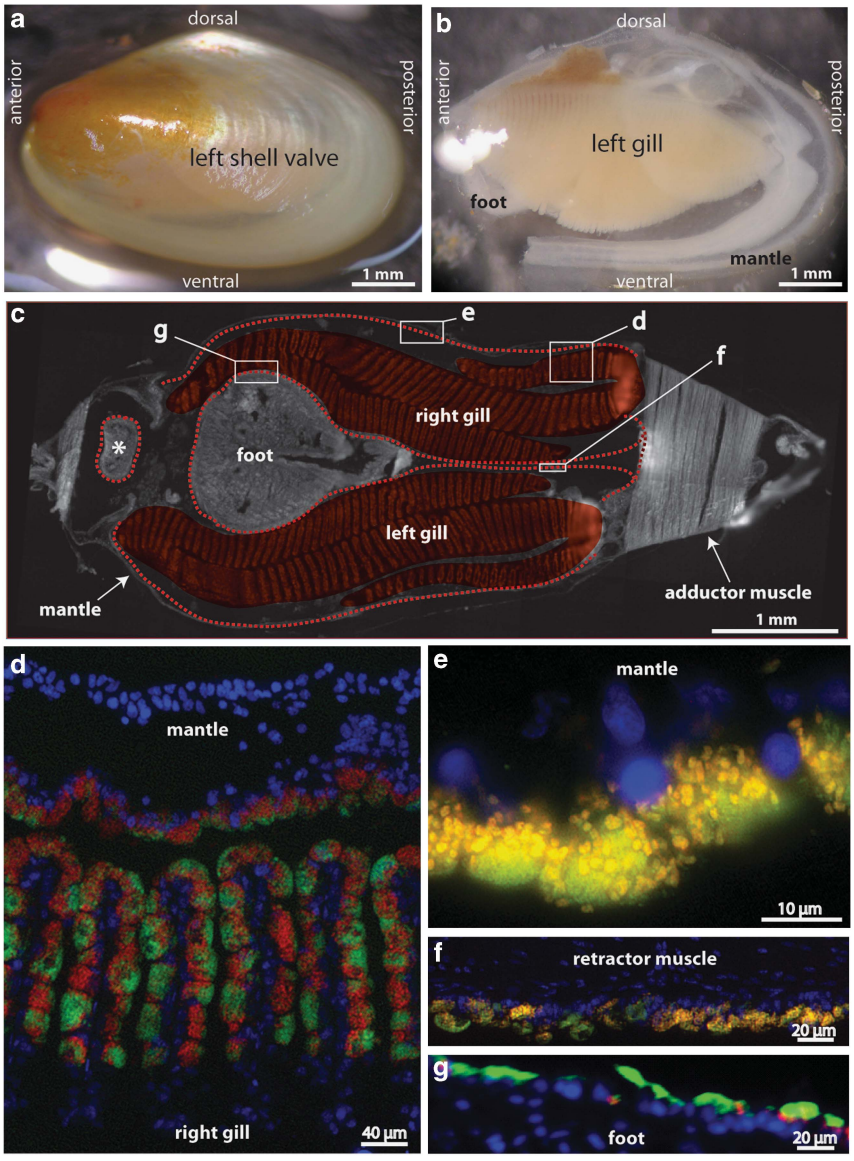

Figure 1 Symbionts colonize many different epithelial tissues in juvenile *B. puteoserpentis* and *B. azoricus*. The mussels are shown in the same orientation in all images with the anterior end on the left and the posterior end on the right. (**a**), (**b**) and (**g**) are *B. azoricus*; (**c**–f) are *B. puteoserpentis*. (**a**, **b**) Lateral view of small juvenile with (**a**) and without (**b**) shell valves. (**c**) Epifluorescence micrograph of a cross section (ventral view) through entire juvenile mussel. The asterisk marks the foot tip that was curled dorsally. Mussel tissue that is colonized throughout the mussel life cycle is colored in light red, whereas mussel tissues that are only infected in juveniles <9 mm are marked with a red dashed line. (**d**–**g**) Symbiont-specific FISH signals of the sulfur-oxidizing symbionts (green) and the methane-oxidizing symbionts (red) in epithelial cells of (**d**) gills and mantle, (**e**) mantle, (**f**) retractor muscle and (**g**) foot. In (**e**) and (**f**) signal overlap in a triple hybridization with the two symbiont-specific probes (red and green) and the eubacterial probe (EUB338 in yellow) makes the methane-oxidizing symbionts appear orange and the sulfur-oxidizing symbionts yellow-green. Owing to differences in signal intensity of the specific probes versus the eubacterial probe, some symbionts appear more yellow than others. The triple hybridization indicates that only the symbionts and no other bacteria are present. Nuclei of the host cells are stained with DAPI (blue).

Table 1 Relationship between shell length and symbiont colonization patterns in *Bathymodiolus* mussels

	B. puteo serpentis (*this study*)		*B. azoricus* (*this study*)		*B. childressi* (*Streams* et al., *1997*)	*B. heckerae* (*Salerno* et al., *2005*)		*B. azoricus* (*Salerno* et al., *2005*)	
Number of specimens	2	6	3	2	24 (in total)	18	4	15	4
Shell length (mm)	6–7	9–21	4–5	27–29	4–8	0.12–8.4	adult (size nd)	0.12–8.4	adult (size nd)
Symbionts in gills	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Symbionts in epithelia besides gills	Mantle, foot and retractor muscles	No	Mantle, foot and retractor muscles	No	Mantle and foot	Mantle	Nd	Mantle	Nd

nd, not determined.

symbiont-specific FISH signals in the epithelial cells of the mantle, foot and retractor muscles (Table 1, Figure 1), but not in other tissues or organs. The distribution and abundance of both symbionts was comparable in all organs except for the much denser colonized gills, based on microscopic observations.

The symbionts are the only bacteria that colonize juvenile host tissues

Extensive FISH analyses of all 13 juveniles showed overlap between the symbiont-specific and general eubacterial probes (as well as with DAPI staining) in all colonized host tissues (Figure 1). This indicates that the sulfur- and methane-oxidizing symbionts are the only bacteria that colonize juvenile mussels.

Larger Bathymodiolus *mussels only have symbionts in their gill tissues*

In contrast to *B. azoricus* and *B. puteoserpentis* juveniles $\leqslant 7$ mm, larger juveniles of both species (9–29 mm) had symbiont-specific FISH signals only in their gills (Supplementary Figure S1). To examine if this spatial restriction of colonization to the gills is also maintained in adult *Bathymodiolus*, we examined mantle tissues attached to gills dissected from *B. azoricus* ($n = 6$, 55–100 mm). We only observed the symbionts in the gills but never in the mantle.

Discussion and Conclusions

Our study provides the first FISH-based evidence that the symbionts of early-stage *B. azoricus* and *B. puteoserpentis* $\leqslant 7$ mm colonize a wide range of epithelial tissues. Together with earlier studies on juvenile *B. azoricus*, *B. heckerae and B. childressi* (Streams *et al.*, 1997; Salerno *et al.*, 2005), these results indicate a consistent pattern of indiscriminate symbiont colonization of a wide range of epithelial tissues in *Bathymodiolus* $\leqslant 8.4$ mm (Table 1). This is remarkable given that in most symbioses examined to date, infection sites are spatially limited to specific tissues or areas, even in early life stages of the host (Bright and Bulgheresi, 2010). The ontogeny of symbiont colonization has been thoroughly examined in only a few of the many bacteria–eukarya symbioses, and it is possible that wide-spread colonization of many different tissues in early life stages is not restricted to *Bathymodiolus* but may also occur in other hosts. Despite the pervasive infection of *Bathymodiolus* juvenile epithelia, our study shows that colonization is only achieved by the symbionts (although we did not check for the intranuclear bacterial parasite that can also infect these hosts (Zielinski *et al.*, 2009). Clearly, the infection process must be highly regulated and specific to ensure that only the symbionts colonize host cells.

The most obvious explanation for the widespread colonization of host epithelia in juvenile *Bathymodiolus* is that it provides the host with additional nutrition. In bivalves, the gills typically develop after the mantle and foot (Raven, 1958; Streams *et al.*, 1997), and symbionts in mantle and foot epithelia could thus provide the host with nutrients before the appearance of its gills. However, filter-feeding is assumed to be common in *Bathymodiolus* (Pile and Young, 1999; Riou *et al.*, 2010) and could provide juveniles with enough food to make the nutritional contribution from the small numbers of symbionts in mantle and foot epithelia irrelevant.

In both *Bathymodiolus* species investigated in this study from two vent sites separated by almost 3000 km, we observed that juveniles $\geqslant 9$ mm only had symbionts in their gills. This indicates an ontogenetic shift in symbiont colonization from indiscriminate infection of different epithelia to a highly restricted spatial limitation to gill bacteriocytes at a developmental stage between 8.4–9 mm. To our knowledge, such an ontogenetic shift in symbiont distribution patterns has not been previously described in *Bathymodiolus* or any other symbioses. *Bathymodiolus* gills with their greatly enlarged surface areas and cilial ventilation are by far the most efficient tissues for providing the symbionts with the oxygen and reduced compounds they need to gain energy. However, the thin outer layer of non-gill epithelia apparently has sufficient access to oxidants and reductants to harbor symbionts in juveniles < 9 mm, and it is not clear how this would change when mussels become larger. On the other hand, the transfer and distribution of nutrients from the symbionts to the host in foot and mantle epithelia might be hindered because the

symbiont-containing cells in these tissues are not immediately adjacent to the hemolymph lacuna as are the gill bacteriocytes. The cost of harboring symbionts in non-gill tissues might therefore outweigh their nutritional benefit. Alternatively, the immune system of early stage *Bathymodiolus* might not be sufficiently developed to prevent indiscriminate infection (Luna-Gonzalez *et al.*, 2004; Huan *et al.*, 2012). Teasing apart the roles that nutrition and the host immune system have in this ontogenetic shift will provide a better understanding of how bacterial colonization patterns are established, maintained and regulated in animal symbioses.

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