Supporting Online Material

Horizontal endosymbiont transmission in hydrothermal vent tubeworms

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Supplementary Methods

LR-White embedding. For all treatments ribonucleasefree, sterile water was used. For FISH and TEM, specimens were fixed in 4 % paraformaldehyde/0.1 M PBS (pH 7.4) containing 10% (w/v) sucrose at 4° C for 12 hr, rinsed three times for 10 min in 0.1 M PBS, partially dehydrated in an ethanol series of (30%, 50%, 70%) for 15 min each and stored in 70% ethanol at 4° C up to 3 months. Larvae and juvenile specimens were embedded in medium grade LR White resin (British BioCell International) as follows: Samples were further dehydrated to 100% ethanol at room temperature. After 3 x 20 min in 100% ethanol they were transferred into 100% LR White resin and put through 8 changes of resin at room temperature (30 min each) and then left in fresh resin overnight. The samples were then transferred into gelatine capsules and the resin polymerized in a vacuum oven at 50° C for 48 hr.

Sectioning. Every specimen was sectioned completely from anterior to posterior. Paraffin embedded specimens were cut in series of 4 μm thick sections. LR White resin embedded specimens were cut in complete serial sections of alternating semithin (1 μm) for FISH and ultrathin sections (70 nm) for TEM. Spurr epoxy resin embedded specimens were cut in series of 70 nm ultrathin sections for TEM.

Transmission electron microscopy. Ultrathin sections were mounted on formvar coated copper slot-grids, stained with uranyl acetate and lead citrate using a Reichert Ultrastainer and observed with a Zeiss EM 902 transmission electron microscope. One aposymbiotic larva, one symbiotic larva and one juvenile were entirely reconstructed using images of at least every 50^{th} section. A schematic sagittal and a cross drawing of each was made.

Fluorescence in situ hybridization on LR-White semithin sections. LR White sections were mounted on gelatine/chromalaun ($KCr(SO_4)_2 \cdot 12H_2O$) coated glass slides. FISH was performed without etching or removal of resin. The hybridization solution (0.9 M NaCl, 20 mM Tris/HCl pH 8.2, 0.01 % SDS, 20% to 35% formamide) containing the oligonucleotide probes (1ng/ml) was directly applied to the sections and hybridization was performed in a humid chamber at 46° C for 4 hr followed by adequate stringency washes[1] (Supplementary Table 1). Hybridizations were carried out using 3 newly designed Cy3-labelled oligonucleotide probes specific for the symbiont 16S rRNA of *Riftia pachyptila*, *Tevnia jerichonana* and *Oasisia alvinae* (Rif/Tev/Oas) (Table 1).

Supplementary Table 1

Oligonucleotide probes for fluorescence in situ hybridization (FISH)

Bacteria / *Archaea* group-specific probes and
Riftia pachyptila / *Tevnia jerichonana* / *Oasisia alvinae* symbiont-specific probes

probe	specificity	% formamide	probe-sequence	target	reference
NON338	negative control	10%	5'-ACT CCT ACG GGA GGC AGC-3'	16S rRNA	1
EUB338 II	most *Bacteria*	35%	5'-GCA GCC ACC CGT AGG TGT-3'	16S rRNA	2
EUB338III		35%	5'-GCT GCC ACC CGT AGG TGT-3'	16S rRNA	2
EUB338		35%	5'-GCT GCC TCC CGT AGG AGT-3'	16S rRNA	3
ALF968	*Alphaproteobacteria*	20%	5'-GGT AAG GTT CTG CGC GTT-3'	16S rRNA	4
BET42a	*Betaproteobacteria*	35%	5'-GCC TTC CCA CTT CGT TT-3'	23S rRNA	1
GAM42a	*Gammaproteobacteria*	35%	5'-GCC TTC CCA CAT CGT TT-3'	23S rRNA	1
Delta495a	*Deltaproteobacteria*	20%	5'-AGT TAG CCG GTG CTT CCT-3'	16S rRNA	5
Delta495b		20%	5'-AGT TAG CCG GCG CTT CCT-3'	16S rRNA	
Delta495c		20%	5'-AAT TAG CCG GTG CTT CCT-3'	16S rRNA	
CF319a	*Cytophaga* / *Flavobacter* subgroup of *Bacteroidetes*	20%	5'-TGG TCC GTG TCT CAG TAC-3'	16S rRNA	6
Arch915	most *Archea*	20%	5'-GTG CTC CCC CGC CAA TTC CT-3'	16S rRNA	3
RifTO147	*Rif*/*Tev*/*Oas* symbiont	20%	5'-GAT TTC TCC GAG TTG TCC-3'	16S rRNA	this study
RifTO445	*Rif*/*Tev*/*Oas* symbiont	35%	5'-TCC TCA GGC TTT TCT TCC-3'	16S rRNA	this study
RifTO830	*Rif*/*Tev*/*Oas* symbiont	20%	5'-CCC TTA TAA TGA GCC CAA CGG-3'	16S rRNA	this study

Supplementary Table 1 *Archaea*-, *Bacteria*- and bacterial group-specific and symbiont-specific oligonucleotide probes targeting the 16S or the 23S rRNA used for fluorescence *in situ* hybridization. *Bacteria*-, *Archaea*- and group-specific probes were 5' end labelled either with Cy3, Cy5, Alexa 488, or FITC and used to characterize other than the symbiotic phylotype in the tubeworms, and in the developing and juvenile tube. The symbiont-specific probes were 5'- end labelled with Cy3 or Cy5. Group- and symbiont-specific probes were used simultaneously in varying combinations. Three newly designed probes specific for the *Riftia pachyptila* / *Tevnia jerichonana* / *Oasisia alvinae* symbionts and could be unambiguously distinguished from the auto fluorescent background of the worm's tissue at even higher stringencies (given as % formamide in the hybridization buffer).

Supplementary References

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3. Amann, R. I., Krumholz, L. & Stahl, D. A. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *J Bacteriol* **172**, 762-70 (1990).

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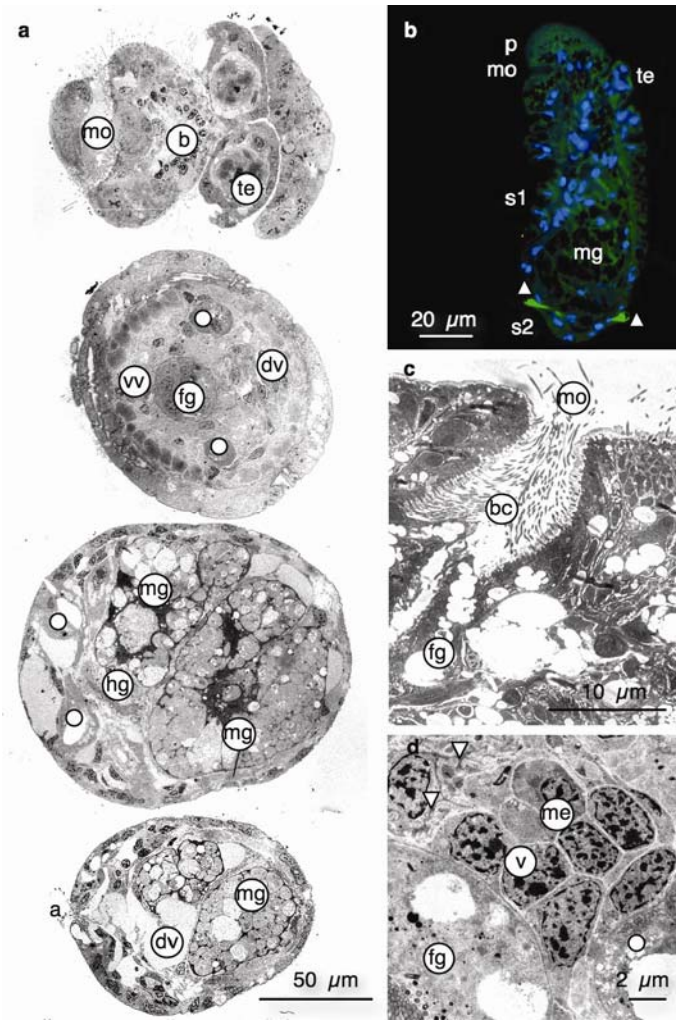
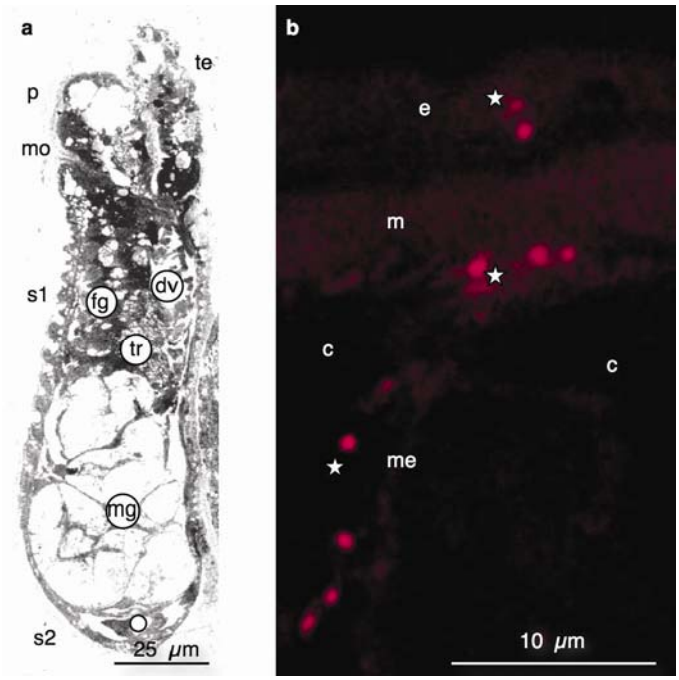
6. Manz, W., Amann, R., Ludwig, W., Vancanneyt, M. & Schleifer, K. Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum cytophaga-flavobacter- bacteroides in the natural environment. *Microbiology* **142**, 1097-1106 (1996).

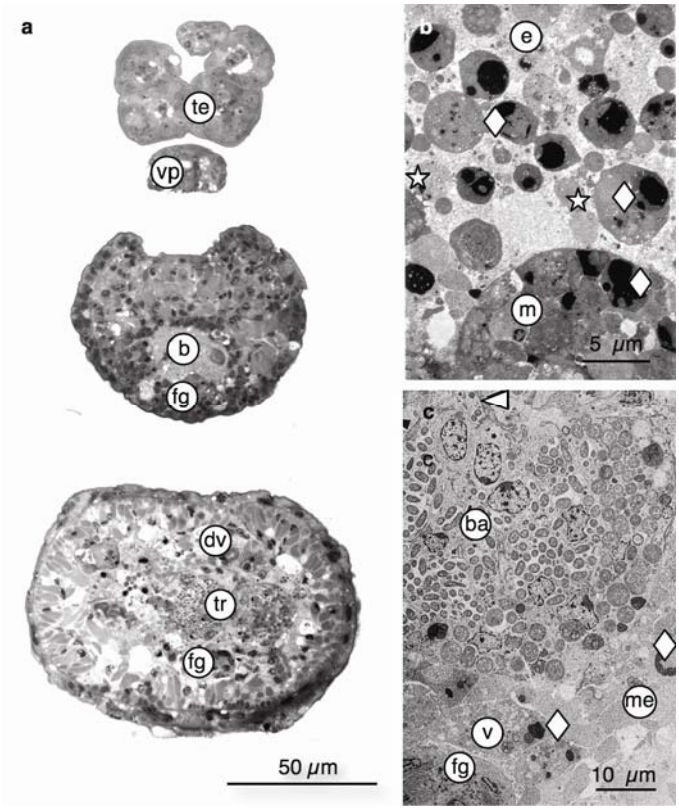
Supplementary Figures



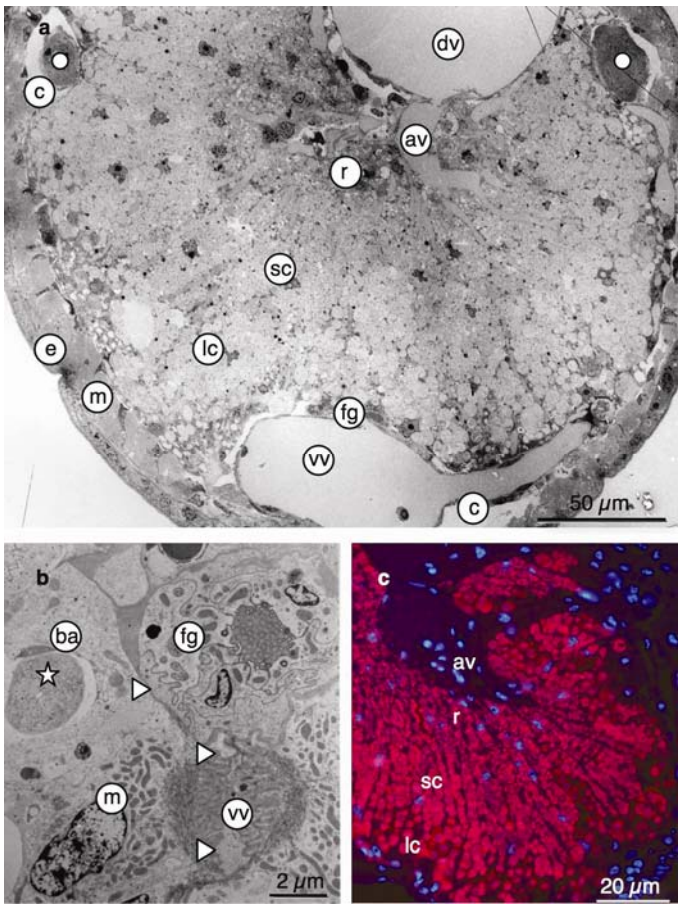
Supplementary Figure 1 Aposymbiotic Vestimentifera larvae (200-250 µm length). **a**, Selected TEM micrographs of cross sections from serial sections of larva (250 µm) in the region of mouth opening (mo) with brain (b) located in the prostomial region and developing 1st pair of tentacles (te); foregut (fg) region with dorsal (dv) and ventral (vv) blood vessels and tube-building pyrifom glands (circles), midgut (mg) and hindgut (hg) region; and posterior end with anus (a) and prominent dorsal blood vessel (dv). **b**, FISH of different specimen (200 µm) on LR White section with symbiont specific probes labelled with Cy3 (red), DAPI counterstain (blue), note the lack of signal of symbiont-specific probes; larva with prostomium (p) mouth opening (mo) in the peristomial region, segment 1 (s1) containing the fore- and midgut (mg) and the first pair of chaetae (triangles) and segment 2 (s2). **c**, Same specimen as in b: TEM micrograph of mouth opening (mo) leading into short buccal cavity (bc) and foregut (fg). **d**, Same specimen as in a: TEM micrograph of area, in which trophosome will develop from mesodermal cells of dorsal mesentery (basal matrix outlined with arrowheads), next to foregut (fg) surrounded by visceral mesoderm (v) next undifferentiated mesoderm (me); circle marks pyriform gland.



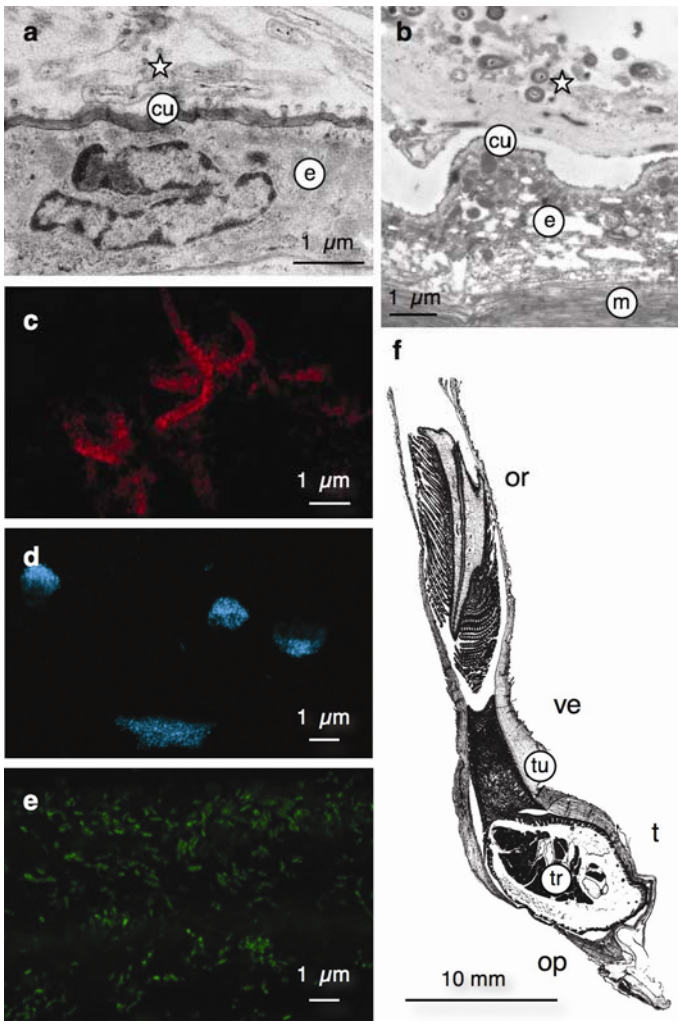
Supplementary Figure 2 Infected Vestimentifera larva (250 µm length). **a**, Light micrograph of sagittal section from serial sections with mouth opening (mo) in peristomial region, anterior located prostomium (p), tentacle (te) arising from segment 1 (s1) followed by segment 2 (s2) with pyriform gland (circle); segment 1 with ventrally located foregut (fg) next to trophosome (tr) and dorsal blood vessel (dv) followed posterior by midgut (mg). **b**, Same specimen as in a: Laser scanning confocal micrograph (LSCM) of FISH; overlay (pink) of two images with symbiont specific probes (red) and universal eubacterial probe mix (blue) showing the rod-shaped symbionts (asterisks) in epidermis (e), muscles (m) and undifferentiated mesoderm cells (me) located in coelom (c).



Supplementary Figure 3 Infected, small juvenile (400 µm length) of Vestimentifera. **a**, Selected TEM cross sections from serial sections in the anterior region showing obturacular region in development with tentacles (te) and peristomial region with ventral medial process (vp) containing foregut and ending in mouth opening; vestimentum with foregut (fg) and brain (b); trunk region with developing trophosome (tr) between dorsal blood vessel (dv) and foregut (fg). **b**, TEM micrograph of small juvenile with symbiont-like bacteria (asterisks) and apoptotic nuclei (diamonds) in the epidermis (e) and muscles (m) of the trunk region. **c**, TEM micrograph of trophosome with bacteriocytes (ba) next to undifferentiated mesoderm (me), arrowhead points to basal matrix of dorsal blood vessel cells; foregut (fg) surrounded by visceral mesoderm (v); note two apoptotic nuclei (diamonds).



Supplementary Figure 4 Symbiotic, large juvenile of Vestimentifera. **a**, TEM micrograph of cross section from serial sections of juvenile (1100µm length) in trunk region showing typical symbiotic organization of tubeworm morphology with epidermis (e), muscles (m), coelomic cavity (c) is restricted to small spaces next to pyriform glands (circles) and ventral (vv) and dorsal blood vessel (dv); reduced foregut (fg) next to extended trophosome filling most of the interior with bacteriocytes containing symbionts with different morphology: rods (r) in the central, small cocci in the median (sc) and large cocci (lc) in the peripheral region followed by a sheath of non-symbiotic cells; fresh blood is brought to the trophosome by extensions of the ventral blood vessel, and blood leaves the trophosome through extensions (axial blood vessel) of the dorsal blood vessel. **b**, TEM micrograph of different animal (1200µm length) with foregut (fg) and ventral blood vessel (vv) with myoepithelial cells (m). The bacteriocyte (ba) – containing the symbionts (asterisk) – next to the foregut is part of the trophosome. Both epithelial cell types (bacteriocyte and myoepithelial cell of ventral blood vessel) share a continuous basal matrix (arrowheads); this organization points to the mesodermal origin of the bacteriocytes. **c,** FISH of different specimen of *Riftia pachyptila* (5 mm length) on LR White section with symbiont specific probes labelled with Cy3 (red), DAPI counterstain (blue), showing the organization of the trophosome lobules in larger juveniles and adult tubeworms with centrally rod-shaped symbionts (r) next to the axial blood vessel (av), followed by small cocci (sc) and large cocci (lc) in the periphery.



Supplementary Figure 5 Developing and solid tube of Vestimentifera **a**, TEM micrograph of aposymbiotic larva; bacteria (asterisk) on surface of cuticle (cu) embedded in developing tube, and epidermis (e). **b**, TEM micrograph of infected larva with developing tube; skin with muscles (m), epidermis (e) cuticle (cu) and bacteria (asterisk) on surface of cuticle. **c-e,** FISH of paraffin section from tube of large juvenile of *Riftia pachyptila* (5 cm). **c,** *Bacteria* probe EUB338 showing thick filaments; **d,** *Cytophaga / Flavobacter* probe showing small cocci; **e**, *Alphaproteobacteria* probe showing rod-shaped bacteria. Using the symbiont-specific probe mix, no label was detected in the tube but symbionts in trophosome were labelled (picture not shown). **f**, Same animal as c-e; sagittal paraffin section showing the general tubeworm organization of anterior obturacular region (or), followed by vestimentum (ve), trunk (t) with trophosome (tr) and posterior opisthosome (op); note the solid tube (tu) surrounding the animal with anterior opening.