Bacterial symbionts in the hepatopancreas of isopods: diversity and environmental transmission

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Abstract

The midgut glands (hepatopancreas) of terrestrial isopods contain bacterial symbionts. We analysed the phylogenetic diversity of hepatopancreatic bacteria in isopod species from various suborders colonizing marine, semiterrestrial, terrestrial and freshwater habitats. Hepatopancreatic bacteria were absent in the marine isopod Idotea balthica (Valvifera). The symbiotic bacteria present in the midgut glands of the freshwater isopod *Asellus aquaticus* (Asellota) were closely related to members of the proteobacterial genera *Rhodobacter*, *Burkholderia*, *Aeromonas* or *Rickettsiella*, but differed markedly between populations. By contrast, species of the suborder Oniscidea were consistently colonized by the same phylotypes of hepatopancreatic bacteria. While symbionts in the semiterrestrial isopod *Ligia oceanica* (Oniscidea) were close relatives of *Pseudomonas* sp. (*Gammaproteobacteria*), individuals of the terrestrial isopod *Oniscus asellus* (Oniscidea) harboured either '*Candidatus* Hepatoplasma crinochetorum' (*Mollicutes*) or '*Candidatus* Hepatincola porcellionum' (*Rickettsiales*), previously described as symbionts of another terrestrial isopod, *Porcellio scaber*. These two uncultivated bacterial taxa were consistently present in each population of six and three different species of terrestrial isopods, respectively, collected in different geographical locations. However, infection rates of individuals within a population ranged between 10% and 100%, rendering vertical transmission unlikely. Rather, feeding experiments suggest that '*Candidatus* Hepatoplasma crinochetorum' is environmentally transmitted to the progeny.

Introduction

Isopods (Crustacea: Isopoda) originated from the marine environment and have successfully colonized terrestrial habitats. It is assumed that the terrestrial suborder Oniscidea shares a common ancestor with the marine suborders Valvifera and Sphaeromatidea (Zimmer, 2002). The midgut glands (hepatopancreas) of the terrestrial isopods *Porcellio dilatatus* (Donadey & Besse, 1972), *Porcellio scaber* (Wood & Griffith, 1988; Hames & Hopkin, 1989; Zimmer & Topp, 1998a, b; Zimmer, 1999; Wang *et al.*, 2004a, b), *Oniscus asellus* (Hopkin & Martin, 1982; Wood & Griffith, 1988; Hames & Hopkin, 1989) (both Oniscidea: Crinocheta), and of the semiterrestrial species *Ligia pallasii* (Oniscidea: Diplocheta) (Zimmer *et al.*, 2001) carry symbiotic bacteria. The hepatopancreas secretes digestive fluids into the hindgut where digestion takes place and is involved in the resorption of digestively released nutrients (Zimmer, 2002). One of the most interesting aspects of bacterial symbionts inside the hepatopancreatic lumen is their proposed contribution to digestive processes of the isopods, i.e. the hydrolysis of cellulose (Zimmer & Topp, 1998a, b; Zimmer *et al.*, 2002) and the oxidation of phenolics (Zimmer, 1999; Zimmer *et al.*, 2002) and lignins (Zimmer & Topp, 1998b; Zimmer *et al.*, 2002). In contrast to macroalgal food sources of marine isopods, detrital food sources of terrestrial (and freshwater) isopods contain little nutrients, but are rich in cellulose and phenolics. Thus, hepatopancreatic bacteria may have facilitated the evolutionary colonization of terrestrial habitats by Oniscidea and their utilization of terrestrial food sources (Zimmer & Topp, 1998b; Zimmer *et al.*, 2001; Zimmer & Bartholmé, 2003).

Either hepatopancreatic bacteria were acquired by the isopods simultaneously with numerous adaptations that allowed colonization of land or the acquisition of hepatopancreatic bacteria was a predisposition to the colonization

of land. In the former case, these bacteria would be lacking in marine isopods; in the latter case, hepatopancreatic bacteria would be present in marine species (Zimmer *et al.*, 2002). In contrast to their terrestrial relatives, those four marine isopod species of the suborders Valvifera and Sphaeromatidea tested thus far do not harbour bacteria in their midgut glands (Zimmer *et al.*, 2001, 2002; S. Fraune & M. Zimmer, unpublished data), even though they live in a benthic environment that is rich in potential bacterial inoculants. Thus, it has been discussed whether these marine isopods either are effective in protecting themselves from bacterial inoculation or lack the capability of hosting bacteria in their hepatopancreas (Zimmer, 2002). However, this is contradicted by the observation of hepatopancreatic bacteria in *Asellus aquaticus* (Zimmer & Bartholmé, 2003), a freshwater representative of Asellota, an isopod suborder common in both marine and freshwater systems. *Asellus aquaticus*, which is one of the most common detritivorous crustaceans in temperate lentic freshwaters, feeds mainly on decaying plant leaf litter like its terrestrial relatives (Isopoda: Oniscidea).

Recently, we reported that two types of phylogenetically distinct hepatopancreatic symbionts – '*Candidatus* Hepatincola porcellionum', curved rod-shaped bacteria related to the *Rickettsiales* (*Alphaproteobacteria*), and '*Candidatus* Hepatoplasma crinochetorum', sphere-shaped *Mycoplasma*-like bacteria (*Mollicutes*) – colonize the epithelial brush border of the hepatopancreas in the terrestrial *P. scaber* (Wang *et al.*, 2004a, b). However, little is known about the identity of bacterial symbionts in other terrestrial isopod species, owing to the failure in isolating and cultivating these bacteria in previous studies. Yet, this knowledge would be valuable in shedding light on the role of bacterial symbionts during the colonization of land by arthropods with marine ancestors.

Many terrestrial arthropods have evolved elegant transmission mechanisms to ensure the inoculation of their progeny with specific bacterial symbionts, involving three different modes of transmission (Cary & Giovannoni, 1993): (1) vertical transmission from (female) parent to the offspring (Aksoy, 2003), (2) horizontal transmission between syntopic hosts and (3) environmental transmission, where the new host generation takes up its symbionts from the environment (McFall-Ngai, 2002). Theory predicts that mutualistic symbioses will evolve under conditions of vertical transmission of the symbiont from parent to offspring (Herre *et al.*, 1999) as the transmission of symbionts is critical in mutualistic symbioses, both for obligate symbionts and for their host. It is only through vertical transmission that a host can ensure that none of its progeny will stay aposymbiotic. Yet, many mutualists rely on horizontal transmission (discussed in Wilkinson & Sherratt, 2001), and horizontal transfer of symbionts is the rule in sexually reproducing animals with symbionts that are not harboured inside the reproductive tract (Douglas, 1995). Thus, knowing the mechanism of symbiont transmission in isopods is crucial to our understanding of isopod–symbiont interactions.

In the present study, we (1) determine the phylogenetic affiliations of symbiotic bacteria in the hepatopancreas of selected isopod species from different habitats (aquatic, semiterrestrial, terrestrial); (2) survey the distribution of the symbionts among geographically distinct isopod populations; and (3) investigate the mode of symbiont transmission.

Materials and methods

Collection and culture of isopods

Idotea balthica Pallas 1772 (Valvifera: Idoteidae) was collected from seaweed at the Falckenstein beach near Kiel, Germany, kept in plastic containers filled with artificial seawater, and fed on brown algae (*Fucus* spp.). *Ligia oceanica* Brandt 1833 (Oniscidea: Ligiidae) was collected beneath rocks at the Falckenstein lighthouse near Kiel, kept in plastic containers with a bottom of 1 cm of wet sand, and fed on brown algae (*Fucus* spp.). *Oniscus asellus* Linnaeus 1758 (Oniscidea: Oniscidae) and *Porcellio scaber* Latreille 1804 (Oniscidea: Porcellionidae) were collected from beneath decaying wood in the botanical garden of the Christian-Albrechts-Universität of Kiel, kept in plastic containers with a bottom of 1 cm of moist plaster, and fed with decaying leaf litter. *Asellus aquaticus* (Linnaeus 1758) (Asellota: Asellidae) was collected in various ponds in the vicinity of Kiel, and in Lake Neuwühren near Plön, about 20 km south-east of Kiel; they were kept in aerated tap water and fed with mixed leaf litter taken from the field. Isopod cultures were maintained at 12 °C.

For the survey of the geographical distribution of bacterial symbionts in different terrestrial isopod species, an area of about 10 m^2 at each collection site was screened for isopods living underneath decaying detritus.

Symbiont transmission

Gravid females of *P. scaber* were divided into treatment and control groups. For the treatment group, isopods were washed five times in autoclaved water, surface-sterilized with 70% ethanol and UV light ($\lambda = 254$ nm) for 10 s and 5 min, respectively, and washed again five times in water. Subsequently, they were cultivated individually in sterile Petri dishes (85 mm diameter) with sterile filter paper (80 mm) covering the bottom. The filter paper was wetted using sterile-filtered supernatant of an aqueous soil suspension and was renewed every 2 days during

the first 2 weeks of the experiment. Leaf litter was surface-sterilized by means of combined ethanol and UV treatment ($\lambda = 254$ nm) before it was offered as food. Gravid females in the control group were also kept individually in Petri dishes, but were not subjected to surface sterilization, and were fed nonsterilized leaf litter. Unfiltered supernatant (see above) was used to moisten the filter papers. Isopods were maintained at 15 °C, 16/8 h light–dark. Isopod hatchlings (mancae) were harvested just before hatching, and juveniles were collected at day 1 (stage 1) and at day 7 (stage 2) after their release from their mother's brood pouch and the immediate removal of the mother upon offspring hatching. For each group, 30 embryos or 40 juveniles were obtained from each of the 10 mothers. Mother isopods were dissected immediately after obtaining their embryos or juveniles.

DNA extraction

Adult isopods (both males and females) were washed five times in autoclaved water, surface-sterilized with 70% ethanol and UV light ($\lambda = 254$ nm) for 1 and 5 min, respectively, and washed again five times in water. After blotting their bodies dry, the midgut glands were dissected and stored in autoclaved reaction tubes filled with 1 mL acetone. Total DNA was extracted and purified using a bead-beating protocol (Friedrich *et al.*, 2001). DNA was recovered through isopropanol precipitation, then rinsed in 70% ethanol (v/v, −20 °C), subsequently resuspended in PCR water (Sigma), and stored at −20 °C before usage.

PCR, sequencing and sequence analysis

The PCR primers applied in this study are listed in Table 1. PCR amplification, cloning, and restriction fragment length polymorphism (RFLP) analysis of 16S rRNA genes followed the protocols described in Wang *et al.* (2004a, b). Diagnostic PCR with symbiont-specific primers used a 200-μL PCR tube with a 50-μL reaction volume containing 200 μM each dNTP, 5.0 μL of a 10 × reaction buffer (Eppendorf), 25 pmol of each primer, 1.0 U of *Taq* DNA polymerase (Eppendorf) and 1 μL of template DNA. Samples were amplified with a DNA thermal cycler (Eppendorf) under the following conditions: initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min, followed by a final elongation for 7 min at 72 °C.

Both strands of the inserts were sequenced using primers M13 forward, M13 reverse, and 533f/907r on an ABI sequencer by GATC (http://www.gatc.de). Sequences were assembled and compared with those in public databases using BLAST (Altschul *et al.*, 1997). Closely related sequences were retrieved and added to the alignment. Only sequences with more than 1200 nucleotides were used for the alignment. Multiple alignments were made using the CLUSTAL W program (Thompson *et al.*, 1994). Alignments were always manually checked. Phylogenetic analysis utilized the maximum-parsimony, neighbour-joining and minimum evolution algorithms as implemented in MEGA 3.1 (Kumar *et al.*, 2004).

The 16S rRNA gene sequences were submitted to GenBank under accession numbers AY447040–AY447042, AY539721–AY539726 and AY573580–AY573582.

Denaturing gradient gel electrophoresis (DGGE)

Denaturing gels were prepared using two 6.5% (w/v) acrylamide gel stock solutions [37.5 : 1, acrylamide-*N*,*N*′′-methylenebisacrylamide in 1 × Tris-acetate-EDTA (TAE)

Table 1. Primers used in this study

Primer	OPD designation*	Primer sequence (5′–3′)	Reference or source
27f	S-D-Bact-0007-a-S-21	CAGAGTTTGATCCTGGCTCAG	Weisburg *et al.* (1991)
63f	S-D-Bact-0043-a-S-21	CAGGCCTAACACATGCAAGTC	Marchesi *et al.* (1998)
533f	S-D-Bact-0515-a-S-19	GTGCCAGCAGCCGCGGTAA	Henckel *et al.* (1999)
907r	S-D-Bact-0907-a-A-15	AATTCCTTTGAGTTT	Henckel *et al.* (1999)
1387r	S-D-Bact-1387-a-A-18	GGGCGGWGTGTACAAGGC	Marchesi *et al.* (1998)
1492r	S-D-Bact-1492-a-A-22	TACGG(C/T)TACCTTGTTACGACTT	Weisburg *et al.* (1991)
PsSym137f	S-G-Hepa-0120-a-S-18†	ACACGTGGGAATTTGGCT	This study
PsSym372f	S-G-Hepa-0352-a-S-21‡	CAGCAGTAGGGAATTTTTCAC	This study
	GC-clamp	CGCCCGCCGCGCCCCGCGCCCGG CCCGCCGCCCCCGCCCCG	

*Except for the GC clamp and primers M13F/M13R, the nomenclature is according to Alm *et al.* (1996).

†Complemented sequences of the specific probe PsSym120 (Wang *et al.*, 2004a) were used as a '*Candidatus* Hepatincola porcellionum'-specific PCR forward primer with the *Bacteria*-specific reverse primer 1492r (Weisburg *et al.*, 1991).

‡Complemented sequences of the specific probe PsSym352 (Wang *et al.*, 2004b) were used as a '*Candidatus* Hepatoplasma crinochetorum'-specific PCR forward primer with the *Bacteria*-specific reverse primer 1492r (Weisburg *et al.*, 1991).

(containing 40 mM Tris base, 20 mM sodium acetate and 1 mM EDTA-Na_2)] with 0 and 80% denaturant [100% denaturant contained 7 M urea and 40% (v/v) formamide in 1 × TAE], respectively. A linear 40–80% denaturant gradient was prepared according to the method of Myers *et al.* (1987).

For PCR amplification of bacterial DNA prior to DGGE, a GC clamp was attached at the 5′ end of the *Bacteria*-specific reverse primer 907r (Henckel *et al.*, 1999). After pre-electrophoresis for 30 min, PCR products were loaded onto the gel, which was run at 60 °C and 60 V for 15 h. The gel was stained with ethidium bromide (0.5 μg mL^{-1}) for 15 min and photographed under UV light with a digital camera.

FISH

Dissected and ruptured hepatopancreas preparations, kept in 500 μL phosphate-buffered saline (PBS; see above), were homogenized by repeated passage through a pipette tip. After gravity sedimentation of the tissue shreds (5 min), 450 μL of supernatant was carefully transferred to a new sterile 1.5-mL tube containing 50 μL of 40% (w/v) formaldehyde. After fixation at 4 °C for 15 h, the samples were centrifuged at 10 000 ***g*** for 5 min and washed three times with 500 μL PBS. The final pellets were dissolved in a mixture of 250 μL PBS and 250 μL of 97% (v/v) ethanol and stored at − 21 °C.

For fluorescence hybridization, hepatopancreas samples were filtered onto polycarbonate filters (0.2-μm pore size) and dried at 46 °C for 30 min. Samples were stained with 4′,6-diamidino-2-phenylindole (DAPI) and hybridized with specific fluorescently labelled oligonucleotides (Table 2) as described by Wagner *et al.* (1993); negative controls with an EUB338 antisense probe (Wallner *et al.*, 1993) were used to exclude nonspecific probe binding. All probes were synthesized and 5′-labelled with the fluorescent cyanine dye Cy3 or with 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester by Thermo Hybaid (http://www.interactiva.de). Samples were covered with Citifluor (Citifluor Ltd, London) and examined at 1000-fold magnification with a Zeiss Axiophot epifluorescence microscope using filter sets for DAPI, Cy3 and fluorescein. Images were recorded with a digital camera (Olympus).

For *in situ* hybridization, thin sections (8 μm) of paraffin-embedded midgut glands, embryos and juveniles were prepared and hybridized as previously described (Wang *et al.*, 2004a, b).

Results

Hepatopancreatic bacteria in isopods from different habitats

Hepatopancreatic bacteria were detected in midgut gland homogenates of almost all isopod species tested by fluorescence microscopy and whole-cell hybridization with the fluorescence-labelled oligonucleotide probe EUB338, and 16S rRNA genes were successfully amplified from DNA extracts of the midgut glands. The only exception was the marine isopod *Idotea balthica*, in which no bacterial cells were observed in DAPI-stained preparations of the 30 individuals tested, and no PCR products were obtained with bacterial primers.

Table 2. Specific fluorescently labelled oligonucleotide probes used in this study

Fluorescence-labelled probes*	OPD designation†	Probe Sequence (5′–3′)	Target site‡ (rRNA position)	Formamide (%)§	Reference
EUB338	S-D-Bact-0338-a-A-18	GCTGCCTCCCGTAGGAGT	16S (338–355)	0–50	Amann *et al.* (1990)
NONEUB	S-D-Bact-0338-a-S-18	ACTCCTACGGGAGGCAGC			Wallner *et al.* (1993)
ALF1B	S-Sc-αProt-0019-a-A-17	CGTTCGYTCTGAGCCAG	16S (19–35)	20	Manz *et al.* (1992)
GAM42a	L-C-γProt-1027-a-A-17 (with competitor BET42a)	GCCTTCCCACATCGTTT GCCTTCCCACTTCGTTT	23S (1027–1043)	35	Manz *et al.* (1992)
PSM G440¶	S-G-Pseu-0440-a-A-15	CCTTCCTCCCAACTT	16S (440-454)	35	Braun-Howland *et al.* (1993)
PsSym352	S-G-Hepa-0352-a-A-21	GTGAAAAATTCCCTACTGCTG	16S (352–373)	0–20	Wang *et al.* (2004a, b)
PsSym120	S-G-Hepa-0120-a-A-18	AGCCAAATTCCCACGTGT	16S (120–138)	20	Wang *et al.* (2004a, b)

*All probes were synthesized and 5′-labelled with the fluorescent cyanine dye Cy3 or with 5 (6)-carboxyfluorescein-*N*-hydroxysuccinimide ester. NONEUB, negative control probe complementary to EUB338; EUB338, most *Bacteria*; ALF1B, *Alphaproteobacteria*, some *Betaproteobacteria*, spirochaetes; GAM42a, *Gammaproteobacteria*; PSM G440, *Pseudomonas* spp.; PsSym352, '*Candidatus* Hepatoplasma crinochetorum' symbionts; PsSym120, '*Candidatus* Hepatincola porcellionum' symbionts.

†The nomenclature is according to Alm *et al.* (1996).

‡*Escherichia coli* numbering (Brosius *et al.*, 1981).

§Percentage amount of formamide (v/v) in the hybridization buffer.

¶No mismatch in the target site of clones Lo-6 and Lo-8.

Hepatopancreatic bacteria in the semiterrestrial *Ligia oceanica*

In the semiterrestrial isopod *Ligia oceanica*, the hepatopancreatic bacteria were straight, rod-shaped cells (0.8 μm wide and 1.8–2.5 μm long: Fig. 1a–c) of uniform appearance. The two ribotypes in the corresponding clone library differed only slightly in their RFLP patterns. Phylogenetic analysis of representative clones showed that they were closely related to each other (99% sequence identity) and represented a lineage within the genus *Pseudomonas* (Table 3). Cell densities ranged from 0.3×10^7 to 6.0×10^7 cells per animal ($n = 10$). All bacteria in the hepatopancreas of *L. oceanica* (DAPI-stained: Fig. 1a–c) exhibited strong hybridization signals with probes EUB338, GAM42a (with BET42a as competitor) and PSM G440, specific for all *Bacteria*, only *Gammaproteobacteria* and members of the genus *Pseudomonas*, respectively, confirming that the cloned 16S rRNA genes originated from the hepatopancreatic symbionts and that the latter form a homogeneous population of *Pseudomonas* sp.

Hepatopancreatic bacteria in *Oniscus asellus* and other terrestrial isopods

Individuals of the terrestrial isopod *Oniscus asellus* contained either spherical cells (0.5–0.8 μm in diameter) or curved rods (0.5 μm wide and 1.5–3.8 μm long), which morphologically resembled the two previously described bacterial symbionts in the hepatopancreas of the closely related isopod species *Porcellio scaber* (Wang *et al.*, 2004a, b). Cell densities ranged between 0.6×10^8 and 9.3×10^8 cells per animal ($n = 5$).

Accordingly, DGGE of PCR products obtained with the same primer combination (533f/907r) yielded a single DNA band for *L. oceanica*, whereas two different bands were obtained for different individuals of *O. asellus*. The positions

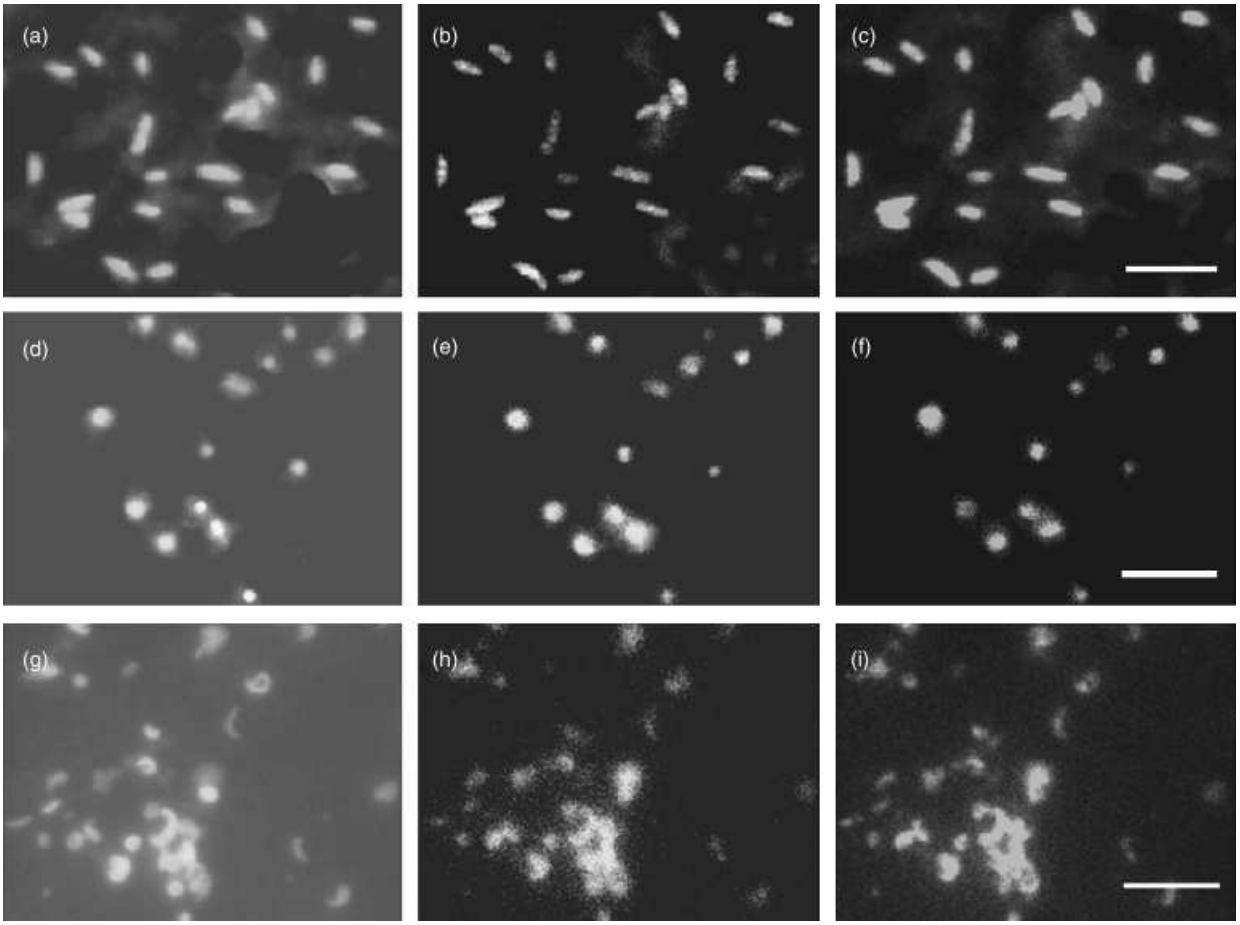

Fig. 1. Epifluorescence microphotographs of symbiotic bacteria in homogenates of the midgut glands of (a–c) the semiterrestrial *Ligia oceanica* and (d–i) the terrestrial *Oniscus asellus* after fluorescence-labelled oligonucleotide probe *in situ* hybridization. (a) Stained with DAPI; (b) hybridized with probe GAM42a specific for *Gammaproteobacteria*; (c) hybridized with probe PSM G440 specific for the genus *Pseudomonas*; (d) left panel: stained with DAPI; (e) probe EUB338 specific for *Bacteria*; (f) probe PsSym352 specific for '*Candidatus* Hepatoplasma crinochetorum'; (g) stained with DAPI; (h) probe ALF1b specific for *Alphaproteobacteria*; (i) probe PsSym120 specific for '*Candidatus* Hepatincola porcellionum'. Scale bar: 5 μm.

Table 3. Ribotypes of clones in clone libraries of hepatopancreatic bacteria in different isopod species, and phylogenetic affiliation of representative clones of each ribotype

Isopod species	Clones tested	Clones in ribotype	Representative clone ID	Sequence affiliation		Sequence similarity (%)
				Subclass	Closest relative	
*Asellus aquaticus**† (Asellota: Asellidae) (Plön)	15	8	Aa-12	*Alphaproteobacteria*	*Rhodobacter* sp. (AY584573)	98.1
		7	Aa-3	*Gammaproteobacteria*	*Aeromonas sobria* (X74683)	99.9
*Asellus aquaticus**† (Asellota: Asellidae) (Kiel)	37	15	Aa-7	*Gammaproteobacteria*	Uncultured *Gammaproteobacterium* (AY947958)	98.8
		13	Aa-10	*Gammaproteobacteria*	Uncultured *Gammaproteobacterium* (AY947958)	98.9
		9	Aa-11	*Betaproteobacteria*	*Burkholderia* sp. N2P5 (U37342)	99.9
Ligia oceanica‡§ (Oniscidea: Ligiidae) (Kiel)	33	16	Lo-6	*Gammaproteobacteria*	*Pseudomonas* sp. (AY958857)	99.7
		17	Lo-8	*Gammaproteobacteria*	*Pseudomonas* sp. (AY958857)	99.5
Oniscus asellus†¶ (Oniscidea: Oniscidae) (Kiel)	12	6	Oa-2	*Mollicutes*	'*Candidatus* Hepatoplasma crinochetorum' (AY500250)	99.2
		4	Oa-12	*Mollicutes*	'*Candidatus* Hepatoplasma crinochetorum' (AY500250)	98.7
		2	Oa-13	*Mollicutes*	'*Candidatus* Hepatoplasma crinochetorum' (AY500250)	98.7

*Freshwater species.
†Feeds on terrestrial detritus.
‡Semiterrestrial species.
§Feeds on microalgae and macroalgal detritus.
¶Terrestrial species.

of the bands in the acrylamide gel obtained for *O. asellus* were identical to those obtained with clones of '*Candidatus* Hepatincola porcellionum' and '*Candidatus* Hepatoplasma crinochetorum' present in *P. scaber* (Wang *et al.*, 2004a, b). These bacterial species seem to mutually exclude each other, as we never found an individual isopod that harboured both symbiont in its midgut glands.

A clone library of 16S rRNA genes from *O. asellus* comprised three ribotypes that differed only slightly in their RFLP patterns. Representative clones showed high sequence similarity among each other (98.1–99.6%). They were closely related to the sphere-shaped symbiont '*Candidatus* Hepatoplasma crinochetorum' in the hepatopancreas of *P. scaber* (Table 3).

The specific probe PsSym352, previously designed to detect '*Candidatus* Hepatoplasma crinochetorum' in *P. scaber* (Wang *et al.*, 2004b), also matched the target sequence of all three cloned 16S rRNA genes from *O. asellus* (Fig. 1d–f). All of the DAPI-stained, sphere-shaped cells showed specific hybridization with both probe EUB338 and PsSym352, supporting the close relationship between the *Mycoplasma*-like symbionts found in *O. asellus* and *P. scaber*. The curved rod-shaped hepatopancreatic bacteria found in *O. asellus* (Fig. 1g–i) hybridized with the *Bacteria*-specific probe (EUB338), the *Alphaproteobacteria*-specific probe (ALF1B) and the probe PsSym120 designed to detect specifically '*Candidatus* Hepatincola porcellionum' in *P. scaber* (Wang *et al.*, 2004a), which suggested that the second symbiont, which was not represented in the clone library of *O. asellus* (see above), belongs to this rickettsial lineage.

Using the specific FISH probes, '*Candidatus* Hepatoplasma crinochetorum' was detected not only in *O. asellus* and *P. scaber* populations collected in different locations (Table 4), but also in the terrestrial isopod species *Philoscia muscorum* (Scopoli 1763), *Armadillidium vulgare* (Latreille 1804), *Trachelipus rathkii* (Brandt 1833) and *Alloniscus perconvexus* Dana 1854, whereas '*Candidatus* Hepatincola porcellionum' was found only in *T. rathkii*. Aposymbiotic individuals were present in all species (Table 4). Morphologically different, rod-shaped bacteria, not hybridizing with the probes for '*Candidatus* Hepatoplasma crinochetorum' and '*Candidatus* Hepatincola porcellionum', were observed in the hepatopancreas of 6.3% of *A. perconvexus*.

Hepatopancreatic bacteria in the freshwater isopods *Asellus aquaticus*

The hepatopancreatic community in the freshwater isopod *Asellus aquaticus* was more complex than that in the other isopod species. Microscopic inspection had already shown that bacterial morphotypes in the midgut glands differed between both individuals and populations. Short rod-

Table 4. Prevalence of hepatopancreatic bacteria in various populations of terrestrial isopods (Oniscidea) of different geographical origin, and frequency of '*Candidatus* Hepatoplasma crinochetorum' and '*Candidatus* Hepatincola porcellionum', determined by whole-cell hybridization with specific oligonucleotide probes

Isopod species	Geographical origin	Habitat	*N*	Prevalence* (%)	Morphotype†	Frequency (%)‡	
						Hc	Hp
Porcellio scaber	Kiel	Woodland	22	100.0	S, C	59.1	40.9
	Kiel	Artificial salt marsh	70	100.0	S, C	92.8	7.2
	Köln, Germany	Woodland	9	100.0	S, C	88.9	11.1
	Köln, Germany	Grassland	15	100.0	S, C	85.7	14.3
	Vancouver Island, Canada (BC)	Grassland	24	25.0	S, C	20.8	4.2
	Haines Island, Canada (BC)	Coastal grassland	37	13.5	S, C	8.1	5.4
Oniscus asellus	Kiel	Woodland	35	68.6	S, C, R	45.7	8.6
	Köln, Germany	Woodland	14	71.4	S, C	28.5	42.9
	Köln, Germany	Grassland	11	100.0	S, C	80.0	20.0
	Poitiers, France	Woodland	10	90.0	S	90.0	0
Philoscia muscorum	Kiel	Woodland	13	62.5	S	62.5	0
Armadillidium vulgare	Kiel	Grassland	11	10.0	S	10.0	0
Trachelipus rathkii	Kiel	Grassland	8	80.0	S, C	60.0	20.0
Alloniscus perconvexus	Vancouver Island, Canada (BC)	Sandy beach	16	25.0	S, R	18.8	0

n, number of samples investigated.

*Symbionts detected by DAPI staining.

†Morphotypes: C, curved rods; R, straight rods; S, spheres.

‡Symbionts hybridized with oligonucleotide probes PsSym352 and PsSym120, respectively. Hc, '*Candidatus* Hepatoplasma crinochetorum'; Hp, '*Candidatus* Hepatincola porcellionum'.

shaped cells (0.5 μm wide and 1.0–1.3 μm long) with densities ranging from 1.2×10^6 to 2.9×10^6 cells per animal ($n = 6$) and long rod-shaped cells (0.7 μm wide and 1.8–2.7 μm long) with densities ranging between 0.3×10^6 and 1.7×10^6 cells per animal ($n = 4$) were observed in individuals collected in Plön. Sphere-shaped cells (0.3–0.7 μm wide) with densities ranging from 1.6×10^6 to 1.9×10^6 cells per animal ($n = 2$) and short rod-shaped cells in chains (0.4 μm wide and 0.4–0.6 μm long) with densities of 0.7×10^6 cells per animal ($n = 1$) were observed in different individuals collected near Kiel.

A clone library of 16S rRNA genes representing hepatopancreatic bacteria of a pooled population of eight individuals collected in Kiel contained three different ribotypes (Table 3). Sequencing of representative clones showed that 76% of the clones in the clone library were virtually identical in sequence (99.7% similarity) and were related ($>$ 96% similarity) to *Rickettsiella grylli* (Vago & Martoja, 1963; Roux *et al*., 1997), whereas the rest were virtually identical to a free-living *Burkholderia* species in the *Burkholderia glathei* subgroup (Table 3).

For *Asellus aquaticus* collected in Lake Neuwühren (Plön), 16S rRNA genes were successfully amplified only with the alternative primer pair 63f/1387r (Table 1). The corresponding clone library, representing hepatopancreatic bacteria of a pooled population of 15 individuals, contained two different ribotypes closely related either to *Aeromonas sobria* or to *Rhodobacter* species (Table 3).

Transmission of symbionts in the terrestrial *Porcellio scaber*

Whereas all mother isopods were screened positive for '*Candidatus* Hepatoplasma', no symbiotic bacteria were detected by DAPI/FISH staining in the homogenates of 100 *P. scaber* embryos obtained from 10 mothers, either in the sterile treatment or the control group. The absence of hepatopancreatic bacteria was corroborated by the lack of PCR products from DNA extracts of embryos ($n = 30$ each from 10 mothers from each group) from both control and treatment groups as well as of juveniles from the treatment group. By contrast, an amplicon of 1162 bp was obtained from DNA extracts of juveniles from the control group, indicating the presence of '*Candidatus* Hepatoplasma crinochetorum'.

Data from whole-cell hybridization and PCR were further confirmed by the results obtained with thin sections of whole embryos and juveniles. *In situ* hybridization with probes EUB338 and PsSym352 did not detect any bacteria in embryos from both control and treatment groups or in juveniles of the treatment group, whereas hybridization signal with both probes was observed in the hepatopancreas of juveniles from the control group. The prevalence of bacterial symbionts in juveniles of the control group, however, was low (juvenile stage 1, $<$ 20%; stage 2, $<$ 30%) and varied between different cohorts (juvenile stage 1, 0–20%; stage 2, 0–30%).

Discussion

Symbiont–host relationships

The results of this study support the hypothesis that hepatopancreatic bacterial symbionts of isopods have been acquired simultaneously with numerous adaptations that allowed the colonization of land, as discussed by Zimmer *et al.* (2002). No bacteria or bacterial DNA were found in the hepatopancreas of the marine *Idotea balthica* (Valvifera: Idoteidae). Having thoroughly screened a total of four species of the marine suborders Valvifera and Sphaeromatidea (Zimmer *et al.*, 2001, 2002; S. Fraune & M. Zimmer, unpublished data), we suggest that these marine isopods – although living in a microbe-rich environment with a high risk of bacterial infection – do not harbour hepatopancreatic bacteria. All other isopod species investigated contained bacteria in their midgut glands. The presence and identity of hepatopancreatic bacteria coincided nicely with the phylogenetic position of their isopod hosts (Fig. 2).

The reasons for the exclusion of bacteria from the hepatopancreas of marine isopods are not clear. Sleeter *et al.* (1978) proposed the production of antibiotics in the gut of the wood-boring *Limnoria tripunctata*, simply because they could not find bacteria inside the gut lumen [although Zachary & Colwell (1979) found gut microorganisms in this species]. It is possible that antibiotics of endogenous or external origin prevent the colonization of the midgut glands by bacteria in marine isopods. Hellio *et al.* (2000) found antibiotic agents in nine of 16 species of marine macroalgae, and such compounds might still be active in the guts of their consumers (namely isopods or amphipods), preventing bacterial colonization of the hepatopancreas. In such a scenario, the acquisition of hepatopancreatic symbionts in other isopod species (as shown herein) would simply be the consequence of an evolutionary change in feeding habit, replacing a diet of fresh algae that is potentially rich in antibiotics (Hellio *et al.*, 2000) with detritus or microbial epibionts, lacking the antibiotic agents of macroalgal origin. The former is the major food source of *Idotea balthica* and other Valvifera and Sphaeromatidea, while the latter is the preferred food source of *Asellus aquaticus* (see below), *Ligia oceanica* and other oniscid isopods.

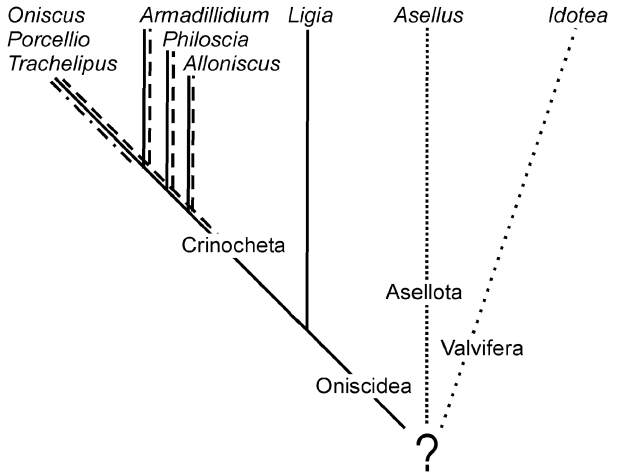

Fig. 2. Phylogenetic relationship of isopod species studied herein (based on Schmidt & Wägele, 2001), overlain with the identity of hepatopancreatic bacteria in the respective species. . . . .: no bacteria, ...: unspecific symbiosis with various bacteria;–: symbiosis with bacteria of the genus *Pseudomonas* (proposed for Crinocheta based on preliminary data); - - -: symbiosis with '*Candidatus* Hepatoplasma', - . - . -: symbiosis with '*Candidatus* Hepatincola'; ?: the phylogenetic relationship between Valvifera, Asellota and Oniscidea is currently debated controversially (cf. Schmalfuss, 1989; Wägele, 1989; Brusca & Wilson, 1991; Tabacaru & Danielopol, 1999).

Plante *et al.* (1990), however, proffer a different explanation, emphasizing the importance of the microhabitat in being a successful host to microbial endosymbionts. While the gut lumen of marine invertebrates exhibits conditions virtually identical with the external environment in terms of ionic and osmotic levels, this is not the case in freshwater invertebrates. The gut of terrestrial invertebrates provides an aquatic habitat in a terrestrial environment. Thus, the significance of microbial gut symbionts can be explained from different points of view: (1) for marine microorganisms, it is not advantageous to colonize the gut of an invertebrate instead of living in sea water, except for being protected from other consumers, whereas the gut of a terrestrial invertebrate grants a more favourable environment than soil or leaf litter; and (2) for terrestrial invertebrates, gut symbionts in a stable environment are more favourable partners to rely on in terms of facilitation in a hostile environment than soil- or litter-colonizing bacteria that inhabit a variable milieu, while this difference is thought to be insignificant for marine invertebrates (Harris, 1993).

Our present results, together with a previous study by Zimmer & Bartholmé (2003), clearly prove the presence of bacterial symbionts in the hepatopancreas of *Asellus aquaticus*. Considering the similar nutritive ecology of freshwater and terrestrial isopods, both being detritivores that feed on leaf litter, the presence of bacteria in the midgut glands may reflect a convergent adaptation to the same food source in different taxonomic branches of isopods (cf. Zimmer & Bartholmé, 2003). On the other hand, Asellota are closely related to, and may even be the sister group of, Oniscidea (cf. Fig. 2). Thus, we may propose a common marine ancestor of these taxa to have acquired the ability to harbour bacterial symbionts in the midgut glands by feeding on food sources other than macroalgae (see above). This evolutionary achievement of hepatopancreatic symbionts may then have been a prerequisite for the utilization of terrestrial food

sources (Zimmer *et al.*, 2001), be it in freshwater or terrestrial habitats. However, hepatopancreatic bacteria in *A. aquaticus* are not only phylogenetically diverse (two or three phylotypes from different proteobacterial lineages), but also differ between isopod populations collected in geographically close sites (Plön and Kiel, Germany). Moreover, the symbionts we detected in *A. aquaticus* are not related to those of terrestrial isopods (Wang *et al.*, 2004a, b), indicating that the midgut glands of terrestrial and freshwater isopods harbour different bacterial species with presumably different relationships to their hosts.

In contrast to *A. aquaticus*, the number of potential hepatopancreatic symbionts appears to be limited to a few bacterial species in Oniscidea. We could unambiguously show that various oniscid isopod species of the Crinocheta (see Fig. 2) from different regions and habitats consistently harboured one of two different bacterial symbionts. '*Candidatus* Hepatoplasma crinochetorum' and '*Candidatus* Hepatincola porcellionum' were present in a total of six and three crinochete isopod species from European and North American locations, respectively. This strongly indicates that symbiotic associations with these two bacterial symbionts are ubiquitous among the Crinocheta (for the semiterrestrial genus *Ligia*, see below). The high incidence (up to 90%) of aposymbiotic individuals, however, indicates a facultative rather than an obligate association between hepatopancreatic symbionts and isopods.

In contrast to the crinochete isopod species tested in the present study, the semiterrestrial *Ligia oceanica* harboured symbionts of the genus *Pseudomonas*. Rod-shaped bacteria were observed also in some individuals of *Porcellio scaber*, *Oniscus asellus* and *Alloniscus perconvexus* (present study; Hopkin & Martin, 1982; Wood & Griffith, 1988; Ullrich *et al.*, 1991; Zimmer *et al.*, 2002); based on FISH, we tentatively classified them as members of the genus *Pseudomonas* (S. Fraune & M. Zimmer, unpublished data).

From our present results on bacterial symbionts in Oniscidea, we conclude that the colonization of supralittoral habitats by the common ancestor of all terrestrial isopods was accompanied by the acquisition of bacterial symbionts. Although a more detailed assay would be required to decide upon this issue unambiguously, we propose that these initial hepatopancreatic symbionts were ancestors of *Pseudomonas* sp. that are still harboured in the semiterrestrial genus *Ligia*, representing an evolutionary prototype of Oniscidea (Schmalfuss, 1978; Carefoot & Taylor, 1995), as well as presumably in at least some crinochete species (see above). These initial symbionts of Oniscidea seem to have been supplemented during the subsequent phylogenetic radiation of terrestrial isopods by new symbionts ('*Candidatus* Hepatoplasma' and '*Candidatus* Hepatincola'), which are currently present in various species of crinochete terrestrial isopods (cf. Fig. 2).

Such an evolutionary takeover of a host has recently been demonstrated experimentally in aphids (Hemiptera: Aphidina), where the primary symbiont, *Buchnera aphidicola*, is being replaced by secondary symbionts (*Gammaproteobacteria*) (Koga *et al.*, 2003). Similarly, tsetse flies (*Glossina* spp.; Diptera: Glossinidae) may lodge a commensal, *Sodalis glossinidius* (*Gammaproteobacteria*), as secondary symbiont that belongs to the same family as the primary symbiont, *Wigglesworthia glossinidia* (Dale & Maudlin, 1999). In dryophtherid beetles (Coleoptera: Curculionoidea) even three lineages of *Enterobactericeae* exist that colonized their host in independent evolutionary steps (cf. Nardon *et al.*, 2003).

Applying a model by Doebeli & Dieckmann (2000), we hypothesize that either the initial acquisition of *Pseudomonas* sp. or the subsequent symbiotic takeover may have led to the branching and later speciation within the Oniscidea, as has been discussed by Buckling & Rainey (2002) in broader context. Leonardo & Muiru (2003) recently presented evidence for nutritional specialization being determined by the identity of facultative bacterial symbionts in aphids.

Hepatopancreatic bacteria in both terrestrial (cf. Zimmer, 2002) and aquatic isopods (Zimmer & Bartholmé, 2003) are thought to contribute to the utilization of low-quality food sources. Notably, members of the proteobacterial genera *Burkholderia*, *Aeromonas* and *Pseudomonas*, close relatives of the symbionts of *A. aquaticus* and *L. oceanica*, are capable of degrading plant polymers, phenolic compounds or polycyclic aromatic hydrocarbons. Recently, a symbiotic *Pseudomonas* sp. in the gut of *Tetraponera* ants (van Borm *et al.*, 2002) was found to be involved in nitrogen fixing. Although nitrogen is abundant in fresh seaweeds, it is significantly reduced in decaying seaweed detritus, one of the major food sources of *Ligia*, and nitrogen-fixing hepatopancreatic symbionts may have helped the semiterrestrial *L. oceanica* and its evolutionary kin in colonizing terrestrial habitats. In the case of the symbionts of terrestrial isopods, however, the next relatives (distantly related members of the *Entomoplasmatales* and *Ricketsiales*) do not offer any clues with respect to their possible function in the symbiosis (Wang *et al.*, 2004a, b). The nature of these associations and their significance for their hosts remain to be elucidated.

Transmission of symbionts

The hepatopancreatic symbionts of terrestrial isopods appear to be transmitted either horizontally among syntopic conspecifics or through the environment. Symbiotic bacteria were not detected in soils and leaf litter samples by FISH (data not shown), but the number of symbionts in the environment – and that of the symbionts necessary successfully to establish the symbiosis in the juvenile host – may be rather low.

In the offspring of *P. scaber* raised in the laboratory under sterile conditions, neither embryos nor juveniles harboured any hepatopancreatic bacteria. However, if kept under nonsterile conditions, bacteria were detected in the midgut glands of juveniles 1 week after hatching. Since we did not surface-sterilize eggs in the brood pouch, we cannot exclude maternal smearing of eggs either inside, or prior to their transport into, the brood pouch. However, if this played a role in symbiont transmission, we would expect embryos and juveniles under sterile conditions to be infected with symbionts, too. As clearly shown by the present results, this is not the case.

The transmission of symbionts is critical in mutualistic symbioses, both for obligatory symbionts and for their host. It is only through vertical transmission that a host can ensure that none of its progeny will stay aposymbiotic, and many obligate symbioses are based on this mechanism. Nevertheless, many mutualists still rely on horizontal transmission (discussed in Wilkinson & Sherratt, 2001), and horizontal transfer of symbionts seems to be the rule in sexually reproducing animals with symbionts that are not harboured inside the reproductive tract (Douglas, 1995): larvae of pony fish (*Leiognathus nuchalis*; Perciformes: Leiognathidae) are aposymbiotic when they hatch and become infected by luminescent bacteria at an age of at least 45 days (Wada *et al.*, 1999). Similarly, aposymbiotic juveniles of lucinid mussels acquire sulfide-oxidizing intracellular gill symbionts (*Gammaproteobacteria*) horizontally.

Given that competition between different symbionts may exert negative effects on the host (discussed in Koga *et al.*, 2003), it seems advantageous to the host to keep the diversity of symbionts low. Again, the best way to achieve this aim is controlled vertical transmission, but even those systems dependent on environmental transmission have developed strategies to master this problem. The flatworm *Convoluta roscoffensis* harbours endosymbiotic algae of the genus *Tetraselmis*, but host individuals differ in what species of *Tetraselmis* they accommodate. Aposymbiotic juveniles take up algae while feeding and reject all but one species (Douglas, 1980). A similar transmission mechanism, with bacteria being ingested along with the food, has been proposed for the sponge *Halichondria panicae* and its symbionts of the genus *Rhodobacter* (*Alphaproteobacteria*) (Althoff *et al.*, 1998; discussed in Stackebrandt & Pukall, 1999; Müller, 1999).

Horizontal transfer of symbionts via faeces or litter, as suggested by our findings in *P. scaber* (see above), seems to be inefficient, since it apparently does not ensure that all offspring gain access to (the correct) symbionts, indicated by the often low prevalence of symbionts in the hosts tested herein. Nonetheless, despite the lack of detection of the symbionts outside of their hepatopancreatic habitat, '*Candidatus* Hepatincola' and '*Candidatus* Hepatoplasma' are present in 10–100% of individuals tested, suggesting that (1) they are common enough in the environment to ensure frequent infection of hosts, and (2) at least one of the symbiotic partners invests in finding, or being found by, an associate. Since the prevalence of symbiotic bacteria is mainly affected by both transmission rate and fitness effects for the host, and transmission rate is potentially low owing to the environmental transmission route, we assume positive effects of this symbiont to its host, but this remains to be tested.

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