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**Symbiotic bacteria in hepatopancreas (midgut glands)
of isopods (Crustacea: Isopoda):
phylogeny, evolution, and distribution**

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1 General introduction

Microorganisms, the most ancient and ubiquitous residents on our planet, comprise the largest component of biodiversity. It is estimated that bacteria have inhabited earth for at least 2.5 billion years (Brocks *et al.*, 1999) and a total of 10^{10} bacterial genes are distributed throughout the biosphere (Stahl and Tiedje, 2002). Accordingly, other organisms, including plants, animals and humans live in a microbial environment and stay in close association with them. The most specialized form of this interaction, symbiosis, has been proven to have a profound impact on both origins and maintenance of biomes and its ecosystems.

1.1 Symbiosis

Symbiosis, originally derived from the Greek word, is simply ‘living together of unlike organisms’. In its narrowest form, symbiosis is often used in sense of mutualism where both partners are mutually beneficial. However, at present, we are going to view it as it was proposed by de Barry in 1879, as defining a wide range of permanent associations between two or more specifically distinct organisms, at least during a part of the life cycle (Brocks *et al.*, 1999). Symbiotic relationship can be divided into three main categories: mutualism, commensalism and parasitism.

1.1.1 Mutualism

Both partners in symbiosis benefit from the relationship. The first descriptions of mutualistic interactions between bacteria and eukaryotic host organisms date back to the ‘Golden Age’ of bacteriology, through the work of scientists including de Bary, Pierantoni, Buchner, Blockmann, and Mereschkowsky (Buchner, 1965; De Bary, 1879; Lanham, 1968; Pierantoni, 1910; Sapp, 2002).

Mutual symbiosis can be found everywhere in our surroundings. Lichens, one successful partnership between fungus and algae or cyanobacteria, are prevalent in nearly

all habitats even in those that are too harsh or limited for most other organisms. Vesicular-arbuscular mycorrhizae or arbuscular mycorrhizae, one mutual symbiosis between plant root and fungi, can be found in nearly all the grasslands and tropical forest trees (Allen *et al.*, 2003). Temperate zone shrubs and trees commonly have associated ectomycorrhizae (Buscot *et al.*, 2000) and over 200 angiosperm species require nitrogen-fixing actinobacteria (Benson, 1988). Legumes, the most important crop in overall economy of biosphere, form root nodule with nitrogen-fixing bacteria (Long, 1996). In case of mutualism between animals and microbes, termites and their intestinal flagellates, ruminants and rumen microbes are good examples (Ohkuma *et al.*, 1999; Russell and Rychlik, 2001). These combinations contribute greatly to critical nutrient cycle movement by facilitating cellulose degradation. In the marine environment, coral reefs are the result of dinoflagellate individuals encysted within animal tissue (Santos *et al.*, 2004). Many aquatic heterotrophs harbor algae within their cells.

The force driving two different species together, of course, is benefit, which makes them superior in struggle for survival. Generally, for microorganisms, the benefits of the association can be a stable protective environment. For the host, symbionts can contribute in many different ways ranging from photosynthesis, chemosynthesis, nitrogen fixing, digestion to protection, etc..

1.1.1.1 Photosynthesis

Ciliates are highly evolved heterotrophic protozoa. *Paramecium bursaria* engulfs unicellular green algae into its cell vacuoles. As a result, it benefits from nutrition photosynthesised by the alga. In return, the alga obtains carbon dioxide produced by its host and is transported to a place where there is ample light (Karakashian, 1975).

1.1.1.2 Chemosynthesis

The marine oligochaete worm *Olavius algarvensis* possesses no mouth or digestive tract, but harbours two different endosymbiotic sulphate-reducing and sulphide-

oxidizing bacteria, γ -Proteobacteria and δ -Proteobacteria, in the epithelium just below the worm's outer surface. The worm has a unique haemoglobin in its blood to bind oxygen and sulphide the bacteria require and depends on organic carbon compounds synthesized by a consortium of these two kinds of bacteria as the main source of their nutrition (Dubilier *et al.*, 2001).

1.1.1.3 Nitrogen fixation

The nitrogen in proteins of every organisms comes ultimately from air. Only few bacteria can break the triple bond in atmospheric nitrogen molecules and make them available to other organisms. Some of these nitrogen-fixing bacteria form partnership with green plants, which provide them with a shelter called root nodule, which meets the low-oxygen environment required by bacteria for reducing nitrogen to ammonia. Plants acquire enough nitrogen from symbiotic bacteria (Long, 1996). Occasionally nitrogen-fixing symbioses occur between bacteria and animals. Wood-feeding termites provide a home for nitrogen-fixing bacteria in their anaerobic hindgut and benefit from the ammonia synthesis by bacteria because the wood consumed by the termites is low in nitrogen (Breznak, 2000).

1.1.1.4 Digestion

In certain invertebrates, symbiotic microorganisms play a crucial role in digestion. One example are the gut symbionts in termites. The diet of termites mainly consists of wood, lignocellulosic plant material, material derived from it, and soil organic matter (Wood and Johnson, 1986). Most of the termites bear a dense and diverse population of cellulose-digesting flagellate protozoa which plays a crucial role in the efficient digestion of cellulose by termite hosts and for their survival on sound wood or cellulose (Cleveland, 1924, 1925).

1.1.1.5 Biosynthesis of essential nutrients

It is widely accepted that mycetocyte between various microorganisms and insect groups have a nutritional basis (Douglas, 1998; Houk and Griffiths, 1980). Nearly all of those insects that possess mycetocytes spend their life cycle on nutritionally poor or unbalanced diets, e.g., phloem sap, vertebrate blood, or wood, and the microorganisms are believed to provide a supplementary source of essential nutrients, primarily essential amino acids, vitamins, and lipids (Douglas, 1998). It was first demonstrated in *Buchnera*-aphid symbiosis, where symbiotic bacteria provide essential amino acids to aphid hosts (Douglas, 1998). These data were further supported by the genome analysis of the pea aphid symbiont, the γ -Proteobacteria *Buchnera* sp. (Shigenobu *et al.*, 2000). The *Buchnera* genome encodes 54 genes involved in amino acid synthesis, but only for the synthesis of essential amino acids that the aphids are unable to synthesize themselves. Consequently, the endosymbiotic bacteria must gain their non-essential amino acids from their insect host (Shigenobu *et al.*, 2000).

Additionally, some symbionts can also act as protector for their hosts. For example, luminescent bacteria inhabiting squid confuse predators (Wilson and Hastings, 1998). Some symbiotic bacteria can produce antibiotics to protect hosts from pathogen.

1.1.2 Commensalism

The term “commensal” comes from the medieval Latin “commensalis”, meaning “at table together”, and generally refers to an association where one member benefits, but the other member is not harmed (Hooper and Gordon, 2001). However, commensalism is also often used to describe the relationship between vertebrate hosts and most members of their indigenous microbial communities colonizing their digestive and absorptive intestinal tracts. However, this implication is generally a reflection of our lacking knowledge about the specific contributions of symbiotic partners, rather than representing an evidence-based conclusion that benefit is truly restricted to one partner (McFall-Ngai and Gordon, 2003).

We inhabit a microbial world and, consequently, become host to a remarkable diverse number of environmentally transmitted extracellular microorganisms. Acquisition of

our microbial partners starts at birth (Favier *et al.*, 2002). In adults, the total microbial population is speculated to outnumber the total number of somatic and germ cells by at least an order of magnitude (Berg, 1996; Savage, 1977). Our gastrointestinal tract houses the densest number of microbial residents, which await for further identification. Currently, it is thought that the intestinal microbiota has 500-1000 different species, with a calculated biomass of about 1.5 kg (Xu and Gordon, 2003). However, most of them are refractory to cultivation *in vitro*.

Our intestinal ecosystem is characterized by highly dynamic and reciprocal interactions among its microflora, epithelium, and immune system. The species composition of commensals varies along the length of the gut, changes as we develop and age, and is influenced by our living environment (Hooper and Gordon, 2001). Recent studies show that the benefits provided by our intestinal commensals are apparent and important. First, commensals, e.g., *Bacteroides thetaiotaomicron*, can facilitate to fortify the epithelial barrier by helping to repair damaged tissue (Hooper and Gordon, 2001). Second, early postnatal colonization of our intestine by commensals is important for the development of our immune system (Moreau and Corthier, 1988; Sudo *et al.*, 1997). Therefore, we become tolerant of a wide variety of microbial immunodeterminates. Additionally, this education appears to reduce allergic responses to food or environmental antigens (Braun-Fahrlander *et al.*, 2002). The increasing prevalence of atopy (tendency to allergy) in Western countries has led to the hypothesis that an overly hygienic life-style has altered the normal pattern of intestinal colonization during infancy and produced a lack of tolerance to otherwise harmless food proteins and inhaled antigens (Strachan, 1989; Wold, 1998). Third, commensals benefit the developing host by providing new metabolic capabilities at critical times during postnatal development, by supplying microbial factors that influence other aspects of host postnatal (Hooper *et al.*, 2001), and/or by affording resistance to colonization by potential pathogens that can not compete with entrenched residents of the microbial community for nutrients (Hooper, 2004).

1.1.3 Parasitism

In parasitic symbiosis, at least one member of the partners benefits from the relationship, the other members get a net negative effect on the fitness. Except for viruses, all known life forms in the world are parasitized by microorganisms including viruses, bacteria, fungi, and protists.

Parasitic microbes live on or in the body of their hosts, from whom they get nourishment, and to whom they do some damages. For example, in one way, they compete for nutrients with their host or obtain nutrients directly from host tissue. In another way, they damage their host by producing substances that are directly or indirectly toxic to host cells (exotoxins or endotoxins). Exotoxins often play a crucial role in the pathogenesis of microbial disease, such as A-B toxins (Merritt and Hol, 1995), proteolytic toxins (Montecucco and Schiavo, 1994), pore-forming toxins (Welch, 2001), and other toxins (Finlay and Falkow, 1997).

The relative success of the measures and countermeasures taken by parasites and their host affects the dynamics of their association. For humans, it results in illnesses ranging from acute to chronic. For acute illness, the parasite must move on to a new host before it either kills or is killed by, its present host. Acute illness is characterized by causing an acute illness of short duration, being contagious for only a brief period, and a disease ending in the elimination of the parasite (with or without the death of its host). If the host survives, it usually has acquired a lifelong immunity that will prevent reinfection by that parasite. Continued survival of the parasite depends on it quickly finding other susceptible hosts. Influenza, one example of these "hit-and-run" diseases, is a respiratory infection characterized by fever, cough, and severe muscle aches. In the elderly and infirm, it is a major cause of disability and death (often as a result of secondary infection of the lungs by bacteria). Even in the young and healthy, influenza produces a prostrating disease of a few days duration and one not soon forgotten (Glezen, 2004). For chronic infections, the parasite survives for long periods without either killing or being killed by its host.

Besides this classification system, symbiosis can be categorized as obligate or facultative, ecto- (exo-) or endosymbiosis. Like most definitions there are exceptions and often it is difficult to fit a symbiotic association into these simple categories. The degree of association may not be obvious and some organisms may well include more than one type of association in their life cycle. In some cases, the biological function of the symbiont in terms of its contribution to the host, to great degree, has been confirmed. In other cases, the parasitic nature of the symbiont is reasonably clear. Unfortunately, for most non-cultivable symbionts, their beneficial, neutral or deleterious effects on the host remain unknown. Additionally, the symbiont relationship tends to be more complicated once it is subjected to conversion, e.g., transition among parasitism, commensalisms and mutualism.

1.2 Evolution of symbiosis

Traditionally, evolutionary biologists have viewed mutations within individual genes as the major driving force for phenotypic variation leading to adaptation through natural selection, and ultimately generating diversity among species. But, changes in genome repertoire, occurring through gene acquisition and deletion, are the major events underlying the emergence and evolution of bacterial pathogens and symbionts (Ochman and Moran, 2001). Most Prokaryotes possess different mobile genetic elements that allow the acquisition, loss or structural alteration of sometimes large regions of the bacterial genome. Horizontal gene transfer mediated by genomic islands, plasmids, transposons and IS elements, and phages plays an important role in the evolution of pathogenic and symbiotic interactions and represents a powerful mechanism by which the outcome of a symbiont-host interaction can be permanently changed (Hentschel *et al.*, 2000). A large set of symbionts and pathogens have undergone massive gene loss (Ochman and Moran, 2001). Phylogenetic analyses show that these microorganisms are derived from ancestors with larger genomes (Maniloff, 1996; Shigenobu *et al.*, 2000; Woese, 1987) and that they belong to large and ancient clades consisting of only pathogens and/or symbionts, e.g., the Mollicutes, the Rickettsiales, the spirochetes, and the Chlamydiae (Ochman and Moran, 2001). Most of these small genome bacteria are obligate intracellular pathogens or symbionts (Casjens, 1998).

1.2.1 Evidence for symbiont-host co-evolution

Clearly, the two organisms are communicating in some way and are regulating each other's gene expression in well-evolved symbiotic association. Among the most intimate and successful symbioses are those between eukaryotic cells and their mitochondria and chloroplasts. Mitochondria possess many features of bacteria (Henze and Martin, 2001). They are of the size of bacteria and have a remnant genome of their own consisting of a loop of DNA enclosed by double membranes. They replicate on their own in the cytoplasm, independently of the host cell. Moreover, mitochondria are sensitive to some antibiotics to bacteria. Mitochondrial genome sequences reveal a single ancestral genome closely related to the Rickettsial subdivision of the α -Proteobacteria, a group of obligate intracellular bacteria (Martin, 2000). After about 2 billion years since free-living α -Proteobacteria became permanent residents in their host cells, the symbiotic union became so strong that many genes of mitochondria migrated into the cell's host nucleus, where they code for proteins bearing signals that ensure that they target back to the organelles (Jacobs *et al.*, 1983; Martin, 2000).

Recently, phylogenetic analyses based on small subunit rDNA (16S or 18S) are routinely used to elucidate evolutionary relationships within both prokaryotes and eukaryotes (Baumann and Moran, 1997). The congruence between host and symbiont phylogenetic trees implies co-speciation and synchronous diversification. Evidence for cospeciation between prokaryotic endosymbionts and their insect hosts have been previously obtained for *Buchnera*-aphids (Clark *et al.*, 1999; Munson *et al.*, 1991), *Wigglesworthia*-tsetse flies (Chen *et al.*, 1999), *Blochmannia*-carpenter ants (Sauer *et al.*, 2000), *Blattabacterium*-cockroaches (Bandi *et al.*, 1995), and candidate bacterial species *Carsonella ruddii*-Psyllids (Thao *et al.*, 2000) systems. In addition, cospeciation has been observed between gall-forming marine bacteria and algae (Ashen and Goff, 2000), between chemoautotrophic bacteria and deep sea clams (Peek *et al.*, 1998), between luminous bacteria *Vibrio* and sepiolid squids (Nishiguchi *et al.*, 1998), and between mutualistic *Wolbachia pipientis* and nematodes (Bandi *et al.*, 1998).

The ‘degeneracy’ of parasites also supports the theory of co-evolution. During the course of adapting to conditions in their host, parasites often lose structures and functions that were essential for their ancestors or free-living relatives.

All of these studies indicate that, in each case, a single ancient infection was followed by co-speciation over millions of years, with symbiotic bacteria diverging in parallel with their hosts.

1.2.2 Theory of co-evolution

Bacteria are the most ancient residents on Earth. As a result, the appearance and evolution of any later appearing species was affected and shaped by microbes. However, little is known about how coevolution with microorganisms has guided the genomes of the involved partners. A plausible hypothesis is that what begins as a parasitic relationship might over the course of time evolve into a mutualistic one as the two organisms evolve to minimize the damage to the host (Price, 1991; Steinert *et al.*, 2000). This shift sounds rational since the benign strains of *Escherichia coli*, a normal resident of the mammalian intestinal flora, gain pathogenic properties once acquisition of a single pathogenicity island (Hacker and Kaper, 2000). This hypothesis is supported by observation in nature, too. Jeon *et al.* (1976) discovered a culture of amoebas that had become infected with bacteria. The infection slowed down their rate of growth and made them much more fragile. Five years later, the amoebas still were infected but now no deleterious effects could be seen. Most interestingly, the amoebas – or at least their nuclei – had become dependent on the bacterial function (an enzyme produced by the bacteria but no longer by the host). What started as parasitism had evolved into mutualism (the bacteria could not be grown outside their host).

Recent progresses have revealed that the molecular mechanisms that mediate communication between, and cellular modulation of, the involved partners are quite similar in symbiotic and pathogenic interactions (Hentschel *et al.*, 2000). The most notable mechanisms include quorum sensing (Fuqua *et al.*, 1996) and the two-component regulatory systems (Hoch and Silhavy, 1995), which allow adaptation to the constantly changing conditions found in new niches. Moreover, pathogens and possibly

also symbionts are able to modulate the host environment by type III secretion (Hueck, 1998) of effector molecules that interfere directly with host cellular functions (Hentschel *et al.*, 2000). Thus, the underlying strategies of bacteria-host interactions are remarkably similar in pathogens and symbionts, albeit with modified properties and functions to suit individual needs, suggesting an evolutionary conversion from parasitism to mutualism.

Anyway, it is postulated that lateral gene transfer occurred in the very early soup of organisms (Hoffmeister and Martin, 2003). We have no doubt that it happens in the whole evolutionary history of the biosphere including today. The understanding of symbiotic relationships, from the evolution of species to the dynamic relations between microorganisms and hosts, will reveal new insights into the essence of biology.

1.3 Hepatopancreatic symbionts in isopod

Isopod (Crustacea: Isopoda) originated from marine environments but successfully colonized semi-terrestrial, terrestrial and freshwater habitats. Most terrestrial and freshwater isopods mainly feed on plant litter (Zimmer, 2002). Consequently, the successful colonization of land by a common marine ancestor concerns adaptation to food shift from marine algae to terrestrial detritus (Zimmer, 2002). The feeding and nutrition of terrestrial isopods thus are important to understand the evolution of this animal.

The hepatopancreas (midgut glands), lying freely in the body cavity, consists of three or two pairs of tubular caeca in semi-terrestrial and terrestrial isopods, respectively (Hames and Hopkin, 1989; Hopkin and Martin, 1982; Schmalzfuss, 1978). It functions in secreting digestive enzymes and the absorption of nutrients (Zimmer, 2002). Several authors have observed dense numbers of bacteria colonizing the lumen of the hepatopancreas of *Porcellio scaber* (Hames and Hopkin, 1989; Wood and Griffith, 1988; Zimmer and Topp, 1998a, b, 1999), *Oniscus asellus* (Hames and Hopkin, 1989; Hopkin and Martin, 1982; Wood and Griffith, 1988), and *Ligia pallasii* (Zimmer *et al.*, 2001). It is suggested that these bacteria be involved in the hydrolysis of cellulose

(Zimmer and Topp, 1998b) and the oxidative breakdown of lignins (Zimmer and Topp, 1998a) and tannins (Zimmer, 1999). Therefore, hepatopancreatic bacteria of terrestrial isopods might contribute their isopod host's digestion of leaf litter (Zimmer, 2002) which consists mainly of cellulose, lignin and other phenolics with low nutrient contents (Breznak and Brune, 1994). The relationship between hepatopancreatic bacteria and isopods may be mutualistic. In addition, in contrast to high numbers of hepatopancreatic bacteria which are described to contribute to cellulose hydrolysis in the prototypal oniscid, *Ligia pallasii* (Zimmer *et al.*, 2001; Zimmer *et al.*, 2002), no such bacteria were found in the intertidal isopod species of the suborders Valvifera, *Idotea wosnesenskii* and Sphaeromatidea, *Gnorimosphaeroma oregonense* (Zimmer *et al.*, 2001). Accordingly, the acquisition of hepatopancreatic bacteria might have aided the terrestrialization of isopod from the evolutionary point of view (Zimmer, 2002). Therefore, it is important to isolate as well as characterize the biological features of these bacteria. However, all cultivation efforts have been unsuccessful so far.

Presently, PCR-based amplification and phylogenetic analysis of bacterial 16S rRNA genes have proved to be powerful molecular tools for the identification and characterization of uncultivated microorganisms (Amann *et al.*, 1995). As outlined in Figure 1, in order to identify hepatopancreatic bacteria, total DNA was extracted from the homogenized isopod hepatopancreas; based on PCR, 16S rRNA genes or gene fragments were selectively amplified from mixed DNA extract; standard molecular techniques were employed to obtain a clone library and to retrieve 16S rDNA sequence information; and a comparative analysis of the retrieved sequences was performed. This gives information on the identity or relatedness of new sequences in comparison with the available databases and gives a minimal estimate of the genetic diversity in the examined sample. In order to proof that the cloned sequences originated from the bacterial cells thriving in the host, sequence-specific fluorescence-labelled oligonucleotide probes were designed to hybridize with the cells in situ in the hepatopancreas of isopod. As a result, the 16S rRNA approach provides a cultivation-independent alternative to the established techniques for the identification of new microorganisms.

1.4 Aims of the present study

This work aims at (1) molecular identification of bacterial symbionts in hepatopancreas of isopods, (2) symbiotic evolution and association of hepatopancreatic symbionts and isopod hosts, (3) geographic distribution of hepatopancreatic symbionts among natural populations of terrestrial isopods, (4) and environmental transmission of hepatopancreatic symbiont in terrestrial isopod *Porcellio scaber*.

In chapters 2 and 3, the phylogeny, species composition, location, and ultrastructure of the symbiotic bacteria in the hepatopancreas of *Porcellio scaber*, the Common Woodlouse, were investigated. In order to phylogenetic identification of the symbionts, PCR-based clone libraries for bacterial 16S rRNA genes were constructed. Subsequently, based on comparative sequence analysis of cloned 16S rRNA genes with sequences in public database, the phylogenetic affiliations of hepatopancreatic symbionts were retrieved. The cloned sequence-specific oligonucleotide probes were designed to confirm that PCR amplification was free of contamination and to reveal the species composition of the symbionts. Both electron microscopy and thin section hybridisation were used to elucidate the location as well as the cell ultrastructures of the symbionts in hepatopancreas. In parallel, cloning work was assessed by denaturing gradient gel electrophoresis analysis of the DNA extract in order to exclude PCR bias. Thus, these studies will reveal the classification, colonization and diversity of the hepatopancreatic symbionts, which are crucial to understand symbiotic association and evolution as well as to arrange subsequent studies.

In chapter 4, to understand the evolution background of symbiosis, phylogenetically diverse isopods were screened from marine, semi-terrestrial and terrestrial habitats for bacterial symbionts in their hepatopancreas by means of 16S rRNA gene sequencing and fluorescence in situ hybridisation with probes specific for different bacteria. This study could be valuable in shedding light on both the importance of symbionts in digestion as well as on the colonisation of land by marine arthropods.

Hepatopancreatic symbionts in the freshwater isopod, *Asellus aquaticus*, were identified by cloning, sequencing, and phylogenetic analysis of bacterial 16S rDNA (chapter 5).

This study may be of significance with respect to our understanding of isopod phylogeny. Comparative analysis of the phylogeny of hepatopancreatic symbionts in freshwater and terrestrial isopods would either hint on a close phylegentic relationship of these taxa, i.e., on a common marine ancestor that harboured hepatopancreatic symbionts, or might just be a convergent adaptation of freshwater isopod to feeding on leaf litter.

In chapter 6, an extensive and systematic survey for the geographic distribution and infection frequency of the symbiotic bacteria (identified in chapters 2 and 3) in different terrestrial isopod species was performed using specific fluorescence-labelled oligonucleotide probe hybridisation and diagnostic PCR techniques. This investigation will reveal intra- and inter-specific distribution profile of these symbionts, and provide valuable insights into the evolution of the symbiosis.

Transmission mechanism of hepatopancreatic bacterial symbionts in the terrestrial isopod *Porcellio scaber* was explored in chapter 7. In this study, the presence of symbionts in embryo and two early stages of juveniles of *P. scaber* were monitored by using molecular techniques in two groups of isopod mothers cultivated in unsterilized conditions vs. sterilized conditions to elucidate the mode of symbiont transmission from generation to generation. These data further facilitate us to understand symbiosis evolution and association.

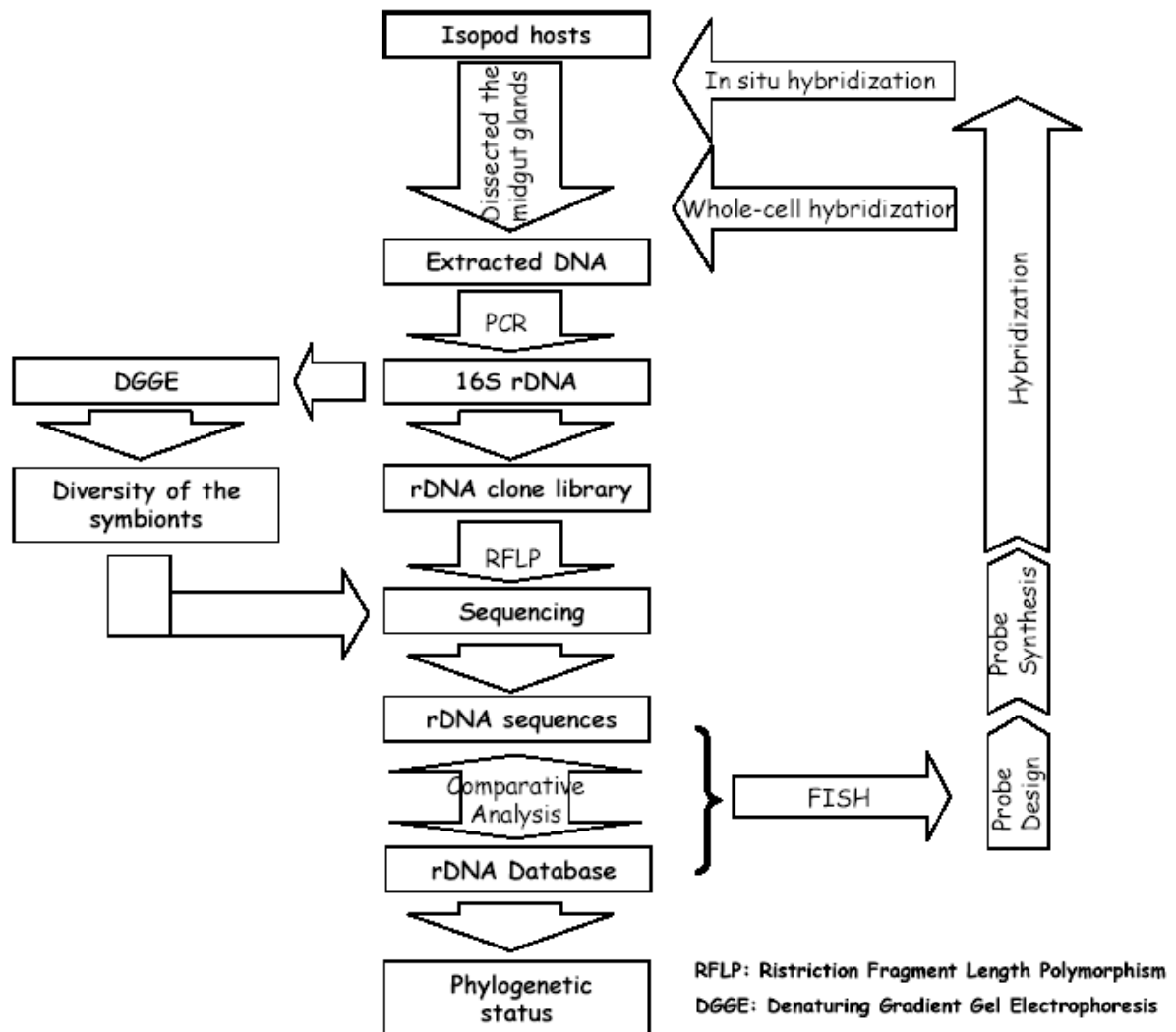


Figure 1 Characterization of uncultivated hepatopancreatic bacterial symbionts by comparative 16S rRNA gene sequence, 16S rRNA-targeted oligonucleotide probe in situ hybridisation, and DGGE analyses.

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2 ‘*Candidatus Hepatincola porcellionum*’, a new, stalk-forming lineage of *Rickettsiales* colonizing the midgut glands of a terrestrial isopod*

2.1 Abstract

Microbial symbionts of animals account for a prominent fraction of the to-date uncultured microorganisms. One example are the hitherto uncultivated bacteria colonizing the midgut glands (hepatopancreas) of terrestrial isopods. Here, we demonstrate that the microbial symbionts in the midgut glands of the Common Woodlouse, *Porcellio scaber* (Crustacea: Isopoda) represent a novel lineage in the α -subdivision of Proteobacteria. Based on comparative sequence analysis of their 16S rRNA genes, their closest (albeit distant) relatives were among the Rickettsiales, which are intracellular symbionts or pathogens of other animals. Transmission electron microscopy and in situ hybridization with fluorescently labelled oligonucleotide probes revealed a homogeneous population of symbionts intimately associated with the endothelium of the hepatopancreas, which apparently interact with the microvilli of the brush border by means of a stalk-like cytoplasmic appendage. Based on its isolated phylogenetic position and unique cytological properties, the provisional name '*Candidatus Hepatincola porcellionum*' is proposed to classify this new taxon of Rickettsiales colonizing the hepatopancreas of *Porcellio scaber*.

Key words: Crustacea • Isopoda • Hepatopancreas • Symbionts • α -Proteobacteria • Rickettsiales • Prosthecate bacteria

2.2 Introduction

Microbial symbionts of animals account for a prominent fraction of the to-date uncultured microorganisms (Amann et al. 1995). In certain invertebrates, symbiotic

bacteria seem to play a crucial role in digestion (Breznak and Brune 1994, Breznak 2000, Brune and Friedrich 2000) or provide food or essential nutrients to the host (Dubilier et al. 1995, Douglas 1998, Moran and Baumann 2000), while others appear to be parasitic (Goebel and Gross 2001). On the whole, however, the lack of pure cultures has severely hampered both the identification of symbionts and the analysis of the nature of the symbiosis. One example are the hitherto uncultivated bacteria colonizing the hepatopancreas (midgut glands) of terrestrial isopods.

The hepatopancreas of crustaceans functions in both digestion and absorption. In terrestrial isopods, it consists of several pairs of midgut caeca, which are considered the main source of digestive enzymes (Hassall and Jennings 1975, Hames and Hopkin 1989). Although cuticular filters effectively prevent particles from entering the hepatopancreatic caeca (Hames and Hopkin 1989), several authors, using direct bacterial counts or electron microscopy, have observed a dense microbial colonization in the midgut glands of various isopods (*Porcellio dilatatus*: Donadey and Besse 1972; *Porcellio scaber*: Hames and Hopkin 1989, Wood and Griffith 1988, Zimmer and Topp 1998a, Zimmer 1999; *Oniscus asellus*: Hames and Hopkin 1989, Wood and Griffith 1988, Hopkin and Martin 1982; and *Ligia pallasii*: Zimmer et al. 2001). This has led to the hypothesis that at least some of the digestive enzymes produced in the midgut glands might be contributed by microbial symbionts (Hames and Hopkin 1989, Zimmer and Topp 1998a, Zimmer 1999, Zimmer and Topp 1998b). Unfortunately, all attempts to isolate and further characterize the bacteria colonizing the midgut glands have been unsuccessful (Wood and Griffith 1988, Zimmer and Topp 1998a). Here, we report on the phylogeny, location, and ultrastructure of the symbiotic bacteria in the hepatopancreas of *Porcellio scaber*, the Common Woodlouse, which are provisionally classified under a *Candidatus* designation.

2.3 Materials and Methods

2.3.1 Specimens

Porcellio scaber was collected from decaying wood in the botanical garden of the Christian-Albrechts-Universität (Kiel, Germany) in January 2002. Only adult isopods of both sexes were used for the experiments.

2.3.2 DNA cloning and phylogenetic analysis

The midgut glands of five individuals were pooled, and total DNA was extracted and purified using a bead-beating protocol and polyvinylpolypyrrolidone spin-columns (Friedrich et al. 2001). 16S rRNA genes were amplified with a Bacteria-specific primer pair (S-D-Bact-0007-a-S-21 and S-D-Bact-1492-a-A-22; Weisburg et al. 1991) and cloned, grouped by their RFLP patterns, and sequenced. Sequences were aligned and phylogenetically analyzed using the ARB software package (version 2.5b; O. Strunk and W. Ludwig, Technische Universität München; <http://www.arb-home.de>). 16S rRNA gene sequences were compared to sequences in public databases using BLAST (Altschul et al. 1997); closely related sequences were retrieved and added to the alignment. Alignments were always manually corrected. For tree reconstruction, highly variable regions of the 16S rRNA gene sequences and sequence positions with possible alignment errors were excluded by using only those positions of the alignment that were identical in at least 50% of all sequences. Only sequences with more than 1400 nucleotides were used for the alignment. Phylogenetic analysis utilized the maximum-likelihood, maximum-parsimony, and neighbour-joining algorithms as implemented in ARB.

2.3.3 DGGE

16S rRNA gene fragments (~450 bp) were amplified using primer pairs (S-D-Bact-0515-a-S-19 and S-D-Bact-0907-a-A-15 with GC clamp) and separated by DGGE (Henckel et al. 1999), using a linear denaturing gradient (40–80%), a constant voltage of 65 V for 16 h, and a temperature of 60 °C.

2.3.4 DAPI staining, whole-cell hybridization, and FISH

The midgut glands of individual isopods were thoroughly homogenized in 500 μ l PBS, and formaldehyde was added to a final concentration of 4% (wt/vol). After fixation for 14 h at 4 °C, the samples were centrifuged at 10,000 \times *g* for 5 min. Pellets were washed three times in PBS, finally resuspended in 250 μ l PBS plus 250 μ l of ethanol (97%, vol/vol), and stored at –21°C until analysis.

Samples were filtered onto polycarbonate filters (0.2 μ m pore size) and dried at 46 °C for 30 min. DAPI (4',6-diamidino-2-phenylindole) staining and hybridisation with fluorescently labelled oligonucleotides were performed as described (Wagner et al. 1993), including negative controls with an EUB338 antisense probe to exclude non-specific 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 cooled CCD camera.

For FISH, thin sections (6 μ m) of paraffin-embedded midgut glands were prepared as previously described (Dubilier et al. 1995). Hybridisation with the fluorescein-labelled group-specific probe ALF1b and the Cy3-labelled clone-specific probe PsSym120 was performed as described above and also included control experiments to exclude non-specific binding.

2.3.5 Probe design

The specific oligonucleotide probe PsSym120 (Cy3-5'-AGC CAA ATT CCC ACG TGT-3'; *E. coli* position 120–138) was designed using the probe-design function of the ARB software. It targets a variable region of the 16S rRNA molecule, where the clones obtained in this study had the same sequence, but where the sequences of all other α -Proteobacteria in the ARB database had at least four mismatches. Although negative controls performed with *Paracoccus denitrificans* gave no hybridisation signal even under low-stringency conditions (0% formamide), probe PsSym120 was routinely used

at high-stringency conditions (20% formamide for double hybridisation with ALF1b, 20% for double hybridisation with EUB338).

2.3.6 Transmission electron microscopy

Freshly dissected midgut glands were fixed with 3.5% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) for 14 h at 4 °C. After washing with 0.075 M cacodylate buffer for 30 min, the tissue was postfixed with 1% OsO₄ in 0.05 M cacodylate buffer for 2 h at 4°C. After additional washing for 30 min, the hepatopancreas were dehydrated in a graded ethanol series at room temperature and embedded in Agar 100 resin (Agar Scientific Ltd., England). Ultra-thin sections (60 nm) were contrasted with 2.5% uranyl acetate and lead citrate (Reynolds 1963) and analyzed using a Philips CM 10 electron microscope.

2.3.7 Nucleotide sequences

The 16S rRNA gene sequences obtained in this study were deposited with GenBank under accession numbers AY188585 and AY189806.

2.4 Results

2.4.1 Morphology and cell density

The hepatopancreas of *P. scaber* consists of two pairs of tubular midgut caeca, which measure approx. 7–10 mm in length and 200–400 µm in diameter in adult animals. DAPI-stained homogenates of the midgut glands confirmed a dense bacterial colonization, which ranged from 0.3×10^7 to 6.7×10^7 cells per individual ($n = 10$). This corresponds to a cell density of about 2×10^7 cells per mg dry wt., which is slightly lower than the density of microorganisms in the hepatopancreas previously reported for other populations of *P. scaber* (Zimmer and Topp 1998a). The cells were rod-shaped (1.5–3.8 µm × 0.5 µm) and moderately to strongly curved (Fig. 1a).

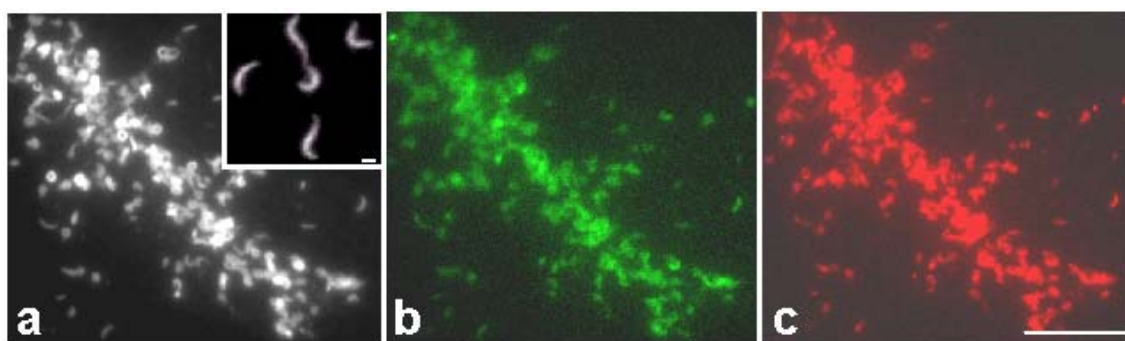


Fig. 1a–c Symbiotic bacteria in a midgut gland homogenate of *Porcellio scaber*. The three epifluorescence photomicrographs show the same microscopic field of a preparation **a** stained with DAPI and double-hybridized with oligonucleotide probes specific for **b** α -Proteobacteria (ALF1b) and **c** the clones obtained in this study (PsSym120), obtained with the respective filter sets. Scale bar, 10 μ m. The insert in **a** illustrates the typical shape of the curved rods. Scale bar insert, 1 μ m.

2.4.2 Cloning and phylogenetic analysis of bacterial 16S rRNA genes

To determine the phylogenetic position of the symbionts, DNA was extracted from isolated midgut glands, and bacterial 16S rRNA genes were amplified using universal bacterial primers and cloned in *Escherichia coli*. Digestion with *Msp*I and *Alu*I endonucleases resulted in two clone groups, comprising 11 and 9 clones, respectively, which differed only slightly in their restriction patterns with each of the two enzymes. The DNA sequences of randomly chosen clones from each clone group were virtually identical (99.7% similarity). Comparison with sequences in public databases gave only low similarities (<81%) to sequences from the α -subclass of Proteobacteria. A detailed phylogenetic analysis revealed that the clones obtained from the hepatopancreas of *P. scaber* represent a novel lineage among the Rickettsiales (Fig. 2). In all phylogenetic trees, reconstructed by neighbour-joining, maximum-parsimony or maximum-likelihood methods, the clones formed an early-branching, monophyletic group only distantly related to *Wolbachia* spp., which include intracellular symbionts of isopods (Bouchon et al. 1998, Cordaux et al. 2001), and to other lineages of α -Proteobacteria.

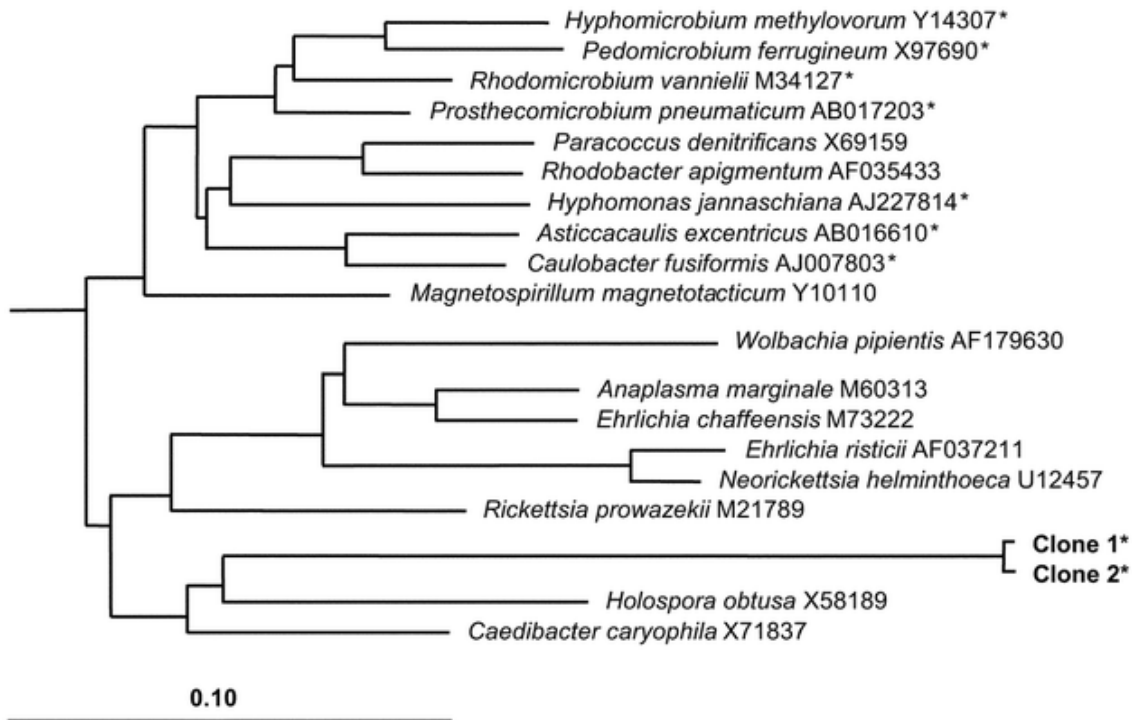


Fig. 2 Phylogenetic tree (maximum-likelihood method) showing the relationship of the 16S rRNA gene sequences of clones 1 and 2 obtained from the midgut glands of *Porcellio scaber* to selected α -Proteobacteria. Stalk-forming representatives are indicated by an asterisk. Selected sequences from other phyla, including stalk-forming Verrucomicrobia (*Prosthecomicrobium* sp.) were used as outgroup. The scale bar indicates an estimated sequence divergence of 0.05 per nucleotide position.

2.4.3 DGGE and FISH analysis of hepatopancreatic symbiotic bacteria

Denaturing-gradient gel electrophoresis (DGGE) of 16S rRNA gene fragments, amplified by PCR from the same DNA extracts using Bacteria-specific primers, resulted in electropherograms that contained only a single band (not shown), which corroborated a low diversity of 16S rRNA genes among the symbionts. To exclude that this homogeneity was due to PCR bias and to confirm that the clones originated from the symbiotic bacteria in the hepatopancreas of *P. scaber*, midgut gland homogenates were subjected to whole-cell hybridisation with fluorescently labelled oligonucleotide probes. In homogenates from 10 different individuals, all DAPI-stained cells (Fig. 1a) always hybridized with the Bacteria-specific probe (EUB338; Amann et al. 1990; not shown), a group-specific probe for α -Proteobacteria (ALF1b; Manz et al. 1992; Fig. 1b), and also

a clone-specific probe designed to detect only the sequences of clones 1 and 2 (PsSym120; Fig. 1c), which indicated that the bacterial symbionts in all individuals tested represent a homogeneous population. To elucidate the exact distribution of the symbionts, thin sections of the midgut glands were hybridized with the same oligonucleotide probes as above. Transverse sections showed that symbiotic bacteria were not spread evenly over the lumen, but were mostly associated with the wall of the hepatopancreatic caeca, which consists of a monolayer of endothelial cells (Fig. 3a–c). Tangential sections substantiated that the bacteria were not located within the cytoplasm, but colonized the surfaces of the epithelium (Fig. 3d–f).

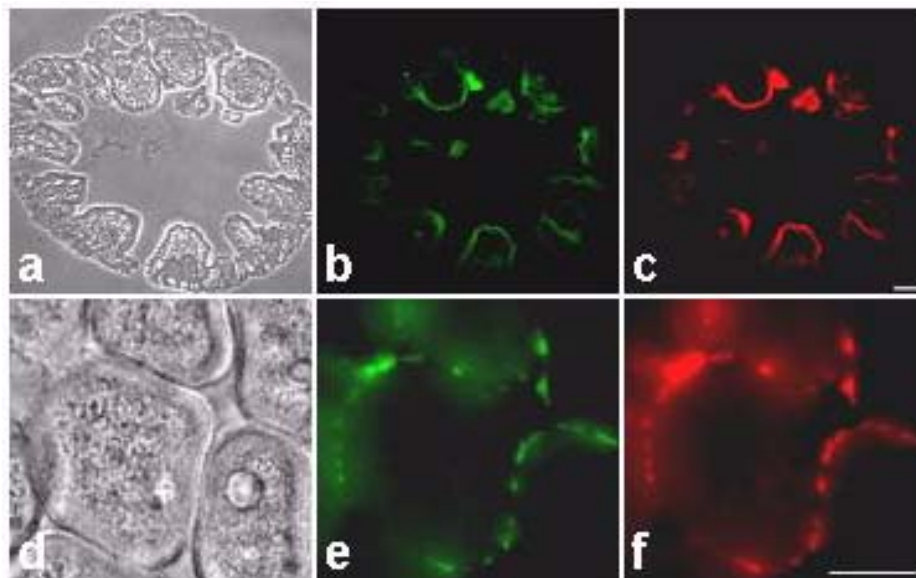


Fig. 3a-f Thin sections of the midgut glands of *Porcellio scaber*, hybridized with fluorescently labelled oligonucleotide probes. The photomicrographs show the same microscopic field of a transverse section (a–c) and a tangential section (d–f), viewed with phase-contrast (a,d) or epifluorescence microscopy, using filter sets for (b,e) probe ALF1b, specific for α -Proteobacteria (Manz et al. 1992), and (c,f) probe PsSym120, specific for the clones obtained in this study. Scale bars: 30 μ m (a–c); 10 μ m (d–f).

2.4.4 An unusual stalk-like appendage of the symbiont revealed by TEM

Also the results of transmission electron microscopy supported that the bacterial symbionts in the hepatopancreas of *P. scaber* consist of a homogenous population.

Ultra-thin sections of the midgut glands showed apparently uniform prokaryotic cells that had a cell wall structure typical of gram-negative bacteria (Fig. 4). However, the cells had two unusual features: a large number of electron-dense inclusions in the cytoplasm and a stalk-like appendage at one cell pole. The inclusions had a regular, cylindrical shape (approx. 150×60 nm) with rounded ends and occurred throughout the cytoplasm, but were never observed in the stalks. The stalks were cytoplasmic protrusions surrounded by a cell wall, had a diameter of 90–100 nm and varied in length (0.7–1.3 μ m). The cells appeared to be oriented with the prosthecae end towards the gland epithelium. In many cases, the stalks were in contact with the brush border, often inserted into the space between the microvilli (Fig. 4b). It remains to be clarified whether the symbionts are in direct contact or interact with the microvilli by means of unknown surface structures, e.g., pili, lipopolysaccharide, or S-layer.

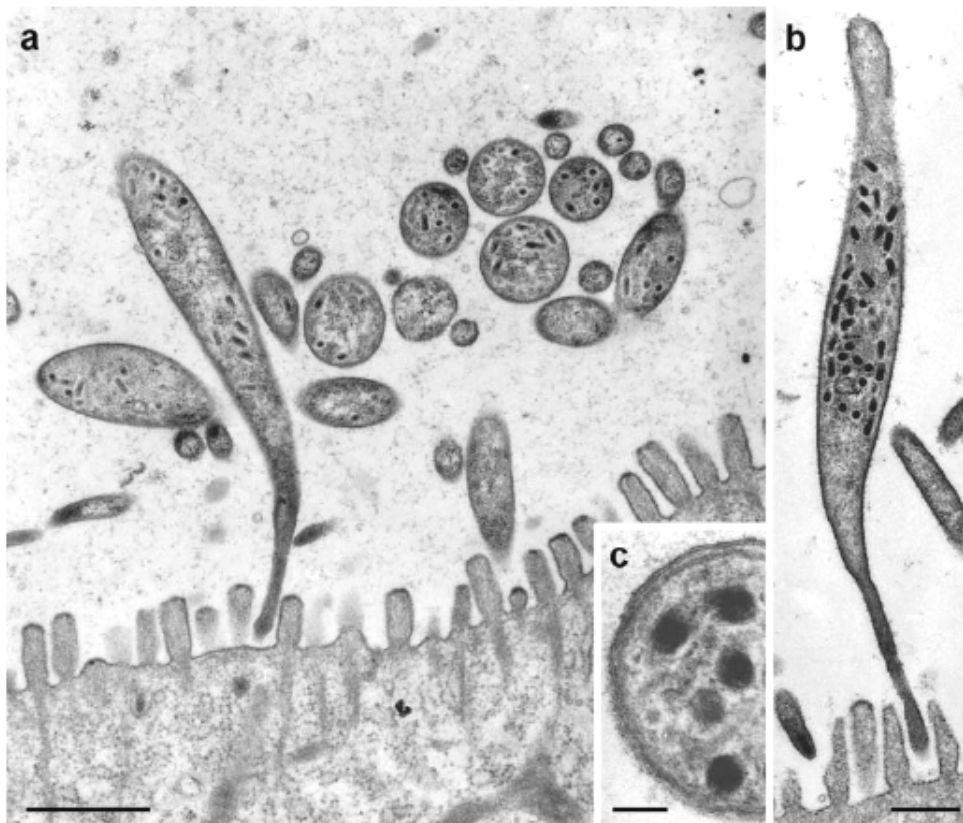


Fig. 4a-c Transmission electron micrographs of bacterial symbionts in the midgut glands of *Porcellio scaber* showing **a** their close association with the epithelium, **b** the frequently observed insertion of the stalk into the gaps between the microvilli of the

brush border, and **c** the typical gram-negative cell wall. Scale bars: 1 μm (**a**); 0.5 μm (**b**); 0.1 μm (**c**).

2.5 Discussion

2.5.1 Phylogeny and evolution of the symbionts

The results of this study document that the bacterial symbionts in the hepatopancreas of *P. scaber* consist of a homogenous population of α -Proteobacteria, representing a novel lineage among the Rickettsiales (Fig. 2). The Rickettsiales comprise five major genogroups, namely Rickettsiae, Ehrlichiae, Neorickettsiae, Wolbachiae and Holosporae (Fredricks 2001), which contain bacteria living as pathogens, symbionts or parasites within the cytoplasm or nucleus of eukaryotic cells. In contrast to these lineages, the bacteria colonizing the hepatopancreatic epithelium of *P. scaber* are extracellular symbionts, although the close association with the epithelium of the host tissue may represent a first step in the evolutionary transition to an intracellular lifestyle.

Intracellular Rickettsiales of the genus *Wolbachia* have been found to infect the reproductive tissues of several isopod species, including *P. scaber* (Bouchon *et al.*, 1998). They are involved in cytoplasmic incompatibility and the feminization of genetic males (Bouchon *et al.* 1998, Stouthamer *et al.* 1999). However, they are not closely related to the symbionts in the midgut glands of *P. scaber* (Fig. 2).

Also the intracellular bacteria reported to invade the hepatopancreas of *P. scaber*, which were initially assigned to the genus *Rickettsiella* based on morphological characteristics (Drobne *et al.* 1999), but later identified as members of the Chlamydiae (Kostanjsek *et al.* 2003), bear little resemblance to the bacteria described in this study. Moreover, the histopathological symptoms associated with the infection were never observed in the course of this study, and the population of isopods from which specimens were taken showed none of the symptoms ascribed to the intracellular pathogens (Drobne *et al.* 1999) even after six months of maintenance in the laboratory.

2.5.2 Appendages of the symbionts

Although an association of microorganisms with the microvilli in the hepatopancreas of *P. scaber* has been previously reported (Hames and Hopkin 1989, Wood and Griffith 1988), the presence of stalks has never been observed. In other prosthecate bacteria (Poindexter 1992), stalk formation is either part of a complex life cycle including a surface-associated phase, or might facilitate nutrient uptake by enlarging the surface-to-volume ratio of the cells. At present, we can only speculate on the function of the prostheca of the symbionts of *P. scaber* – it is possible that the stalked cells represent the sessile stage of an actively proliferating population that may be released into the gut lumen with the digestive fluid, with the surface association preventing washout of the entire population, or that the prostheca functions in the exchange of nutrients between symbiont and host.

In this context, it may be important to note that although division stages of bacteria with inserted stalks were present in *P. scaber* (unpublished TEM photographs), cells were not dispersed throughout the hepatopancreatic lumen and were also never detected in the hindgut lumen receiving digestive fluid from the hepatopancreas by in situ hybridization with clone-specific probes.

Adhesion of microorganisms to epithelial surfaces is characteristic for intestinal habitats and probably the most common type of association between prokaryotes and animals. Not only does attachment prevent washout caused by the flow of digesta, it is also prerequisite for molecular cross-talk between symbionts or pathogens and their hosts and the first step in the invasion of epithelial cells (see, e.g., Klaasen et al. 1992, Lu and Walker 2001, McFall-Ngai 1999, Sansonetti 2002). Since the gap between the microvilli of the hepatopancreatic epithelium of *P. scaber* (50–100 nm) is not big enough to receive the whole bacteria cell, the stalks of the symbionts may represent adaptations to this particular habitat.

The discrepancies among previous morphological descriptions of bacteria in the midgut glands of several isopods (Hames and Hopkin 1989, Donadey and Besse 1972, Wood and Griffith 1988) strongly suggest that different isopod species, and possibly even different populations, are colonized by different symbionts. Since marine isopods

do not contain microorganisms in their hepatopancreas (Zimmer et al. 2002), it has been also speculated that acquisition of such symbionts by the marine ancestors of terrestrial isopods possibly facilitated the nutritional shift from marine algae to terrestrial detritus (Zimmer et al. 2001, Zimmer et al. 2002). Apart from investigating the nature of this peculiar symbiosis, future work also needs to address interspecific and intraspecific differences among the bacteria colonizing the hepatopancreas of terrestrial isopods and their possible significance for physiology and evolution of their host.

As the symbionts in the hepatopancreas of *P. scaber* showed a 16S rDNA sequence similarity to all other members of the α -Proteobacteria of less than 81%, they cannot be assigned to any recognized taxon with this subclass. Following the recommendation of Stackebrandt et al. (2002), who encouraged to use the *Candidatus* concept for well-characterized but as-yet uncultured organisms, provisional classification of the symbionts as '*Candidatus Hepatincola porcellionum*' is proposed.

2.5.3 Description of '*Candidatus Hepatincola porcellionum*'

Hepatincola porcellionum [He.pat.in'co.la. L. n. *hepar* liver *hepatia* (little) liver; L. n. *incola* inhabitant, dweller; N.L. fem. n. *Hepatincola* (a bacterium) inhabiting the liver (hepatopancreas). por.cel.li.o'num N.L. *Porcellio* (Latreille 1804), a genus of isopods; N.L. gen. pl. n. *porcellionum* of (species from) the genus *Porcellio*.]

The short description is as follows: Gram-negative, moderately to strongly curved rods (1.5–3.8 μm \times 0.5 μm); forms stalk-like cytoplasmic appendages at one cell pole; cytoplasm contains numerous electron-dense particles of cylindrical shape. Basis of assignment: 16S rDNA sequence (accession numbers AY188585 and AY189806), hybridization with 16S rRNA-targeted oligonucleotide probe (5'-AGC CAA ATT CCC ACG TGT-3'). Symbiont of *Porcellio scaber* (Crustacea: Isopoda), located exclusively in the lumen of the hepatopancreas; so far uncultivated.

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3 ‘*Candidatus Hepatoplasma crinochetorum*’, a new, stalk-forming lineage of mollicutes colonizing the midgut glands of a terrestrial isopod*

3.1 Abstract

Uncultivated bacteria densely colonizing the midgut glands (hepatopancreas) of the terrestrial isopod *Porcellio scaber* (Crustacea: Isopoda) were identified by cloning and sequencing of their 16S rRNA genes. Phylogenetic analysis revealed that the symbionts represent a novel lineage among the *Mollicutes*, only distantly related (< 82% sequence identity) to members of the *Mycoplasmatales* and *Entomoplasmatales*. Fluorescence *in situ* hybridization with a specific oligonucleotide probe confirmed that the amplified 16S rRNA gene sequences indeed originated from a homogeneous population of symbionts intimately associated with the epithelial surface of the hepatopancreas. The same probe also detected morphotypically identical symbionts in other crinochete isopods. Scanning and transmission electron microscopy showed uniform spherical bacterial cells without a cell wall, sometimes interacting with the microvilli of the brush border by means of stalk-like cytoplasmic appendages, which also appeared to be involved in cell division through budding. Based on isolated phylogenetic position and unique cytological properties, the provisional name '*Candidatus Hepatoplasma crinochetorum*' is proposed to classify this new taxon of *Mollicutes* colonizing the hepatopancreas of *P. scaber*.

3.2 Introduction

The common woodlouse, *Porcellio scaber* (Crustacea: Isopoda), is a cosmopolitan species that feeds mainly on decaying plant material, such as leaf litter, wood, and grass (Zimmer, 2002), which are rich in recalcitrant structural polymers, such as cellulose, hemicelluloses, and lignins (Breznak and Brune, 1994). Together with other terrestrial isopods (Oniscidea), *P. scaber* is considered to play an important role in decomposition processes and nutrient recycling (Zimmer, 2002).

The hepatopancreas (digestive glands) of *P. scaber*, which consists of two pairs of tubular midgut ceca, functions in the secretion of digestive enzymes and the absorption of nutrients. The midgut ceca are densely colonized by microorganisms (Hames and Hopkin, 1989; Wang *et al.*, 2004; Wood and Griffith, 1988; Zimmer and Topp, 1998b; Zimmer, 1999). It has been speculated that hepatopancreatic bacteria, like the gut symbionts of termites and other soil arthropods feeding on fiber-rich diets (Breznak and Brune, 1994; Brune, 2003), might be involved in the digestion of leaf litter, e.g., by producing cellulases or phenol oxidases (Zimmer and Topp, 1998a, b; Zimmer, 1999; Zimmer *et al.*, 2002).

As for many other bacteria associated with invertebrates, all previous attempts to isolate and culture the symbionts *in vitro* have failed, which so far has prevented both the identification of the symbionts and the analysis of the nature of the symbiotic association. In a previous report, we showed that the hepatopancreas of *P. scaber* is colonized by a novel lineage of rod-shaped, stalk-forming symbionts that fell into the alpha subclass of *Proteobacteria*, but were only distantly related to the *Rickettsiales* (<81% sequence similarity) (Wang *et al.*, 2004). In the course of a broader survey of the distribution of such *Rickettsia*-like symbionts among different populations of *P. scaber*, we observed that the hepatopancreas of many specimens contained spherical symbiotic bacteria instead of the rod-shaped forms.

In the present study, we investigated the phylogenetic affiliation, diversity, and location of the spherical symbionts by cloning and sequencing 16S rRNA genes, denaturing gradient gel electrophoresis (DGGE), and hybridization with specific fluorescence-

labeled oligonucleotide probes, and described their morphology and ultrastructure by using scanning and transmission electron microscopy. The results provide novel insights into the origin and diversity of the hepatopancreatic symbionts of isopods.

3.3 Materials and Methods

3.3.1 Specimens

Porcellio scaber was collected from decaying wood in the botanical garden of the Christian-Albrechts-Universität (Kiel, Germany) in August 2002. Only healthy adult isopods of both sexes were used for the experiments.

3.3.2 16S rRNA gene cloning and sequencing

The midgut glands of five individuals were pooled, and total DNA was extracted and purified using a bead-beating protocol and polyvinylpyrrolidone spin-columns (Friedrich *et al.*, 2001). 16S rRNA genes were amplified with a *Bacteria*-specific primer pair (S-D-Bact-0007-a-S-21 and S-D-Bact-1492-a-A-22) (Weisburg *et al.*, 1991) and cloned using the pGEM-T vector cloning system, and transferred into competent *Escherichia coli* (JM 109) cells according to the instructions of the supplier (Promega). Inserts of the expected size (approx. 1,500 bp) were amplified by PCR with M13 forward and M13 reverse primers, and clones were grouped by their restriction patterns obtained after separate digestion with *Msp*I, *Alu*I, and *Rsa*I endonucleases (MBI). For restriction analysis, 18 µl of PCR products, 2 µl of Y⁺/Tango 10× buffer (MBI), and 0.2 µl (1 unit) of the respective enzyme were added into a sterile tube and incubated at 37°C for 15 h. Plasmids were extracted and purified using the Easy Nucleic Acid Plasmid Miniprep Kit 1 (Peqlab), and the inserts were sequenced on both strands using primers 27F (Weisburg *et al.*, 1991), 533F (Weisburg *et al.*, 1991), 907R (Lane *et al.*, 1985), and 1492R (Weisburg *et al.*, 1991) by GATC (<http://www.gatc.de>). Sequences were checked and assembled using the DNASTar software (<http://www.dnastar.com>).

3.3.3 Phylogenetic analysis

Sequences were aligned and phylogenetically analyzed using the ARB software package (version 2.5b; O. Strunk and W. Ludwig, Technische Universität München;

<http://www.arb-home.de>). 16S rRNA gene sequences were compared to sequences in public databases using BLAST (Altschul *et al.*, 1997); closely related sequences were retrieved and integrated into the existing database of aligned sequences of *Mollicutes* with the Fast Aligner function of ARB. These automatic alignments were verified and manually corrected taking into account the constraints of the secondary structure (Ludwig *et al.*, 2004). For tree reconstruction, highly variable regions of the 16S rRNA gene sequences were excluded by using only those positions of the alignment that were identical in at least 50% of all sequences, resulting in a filter that yielded 1368 informative nucleotide positions for construction of the phylogenetic tree. Only sequences with more than 1400 nucleotides were used for the alignment. Phylogenetic analysis utilized the maximum-likelihood, maximum-parsimony, and neighbor-joining algorithms as implemented in ARB (Ludwig *et al.*, 2004).

3.3.4 Denaturing Gradient Gel Electrophoresis (DGGE)

16S rRNA gene fragments (~450 bp) were amplified using primer pairs (S-D-Bact-0515-a-S-19 and S-D-Bact-0907-a-A-15 with GC clamp) and separated by DGGE (Henckel *et al.*, 1999), using a linear denaturing gradient (40–65%), a constant voltage of 65 V for 16 h, and a temperature of 60°C.

3.3.5 DAPI staining and whole-cell hybridisation

The midgut glands of individual isopods were thoroughly homogenized in 500 µl PBS, and formaldehyde was added to a final concentration of 4% (wt/vol). After fixation for 14 h at 4°C, the samples were centrifuged at 10,000 × g for 5 min. Pellets were washed three times in PBS, finally resuspended in 250 µl PBS plus 250 µl ethanol (100%), and stored at –21°C for 1–2 weeks until analysis.

Samples were filtered onto polycarbonate filters (0.2 µm pore size) and dried at 46°C for 30 min. Samples were stained with DAPI (4',6-diamidino-2-phenylindole) and hybridized with fluorescently labeled oligonucleotides as described by Wagner *et al.* (Wagner *et al.*, 1993); negative controls with an EUB338 antisense probe (5'-ACTCCTACGGGAGGCAGC-3') (Wallner *et al.*, 1993) were used to exclude non-specific 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 1,000-fold magnification with a Zeiss Axiophot epifluorescence microscope using filter sets for DAPI, Cy3, and fluorescein. Images were recorded with a cooled CCD camera (Magnafire, INTAS, Germany).

For *in situ* hybridization, thin sections (6 µm) of paraffin-embedded midgut glands were prepared as previously described (Dubilier *et al.*, 1995). Samples were hybridized with the fluorescein-labeled *Bacteria*-specific probe EUB338 (5'-GCTGCCTCCCGTAGG AGT-3') (Amann *et al.*, 1990) and the Cy3-labeled group-specific probe PsSym352 (see below) as described above; control experiments were included to exclude non-specific binding.

3.3.6 Probe design

The group-specific oligonucleotide probe PsSym352 (Cy3-5'-GTG AAA AAT TCC CTA CTG CTG-3'; *E. coli* position 352–372) was designed using the probe-design function of the ARB software. The probe matched exactly the target region of the 16S rRNA molecule of all clones obtained in this study, the *Spiroplasma* group, and the *Mycoplasma pneumoniae* group, but had two or more mismatches to the sequence in all other bacteria in the RDP database, including the *Mycoplasma hominis* group (Cole *et al.*, 2003).

The stringency of hybridization was optimized for probe PsSym352 using hepatopancreatic symbionts as target and *Acidithiobacillus ferrooxidans* as non-target reference strain with 2 mismatches. Formamide concentration in the hybridization buffer was varied between 0% and 35%, while sodium chloride concentrations in the post-hybridization buffer was adjusted accordingly (Lathe, 1985). The fluorescence signal conferred by probe PsSym352 to target cells was stable and similar in intensity at 0 and 10% of formamide, decreased slightly at 20%, and was almost not detectable at 30 and 35% formamide. With non-target cells, there was no signal even under low-stringency conditions (0% formamide). Therefore, we routinely used 10% formamide for single hybridizations and 20% formamide for double hybridizations with EUB338.

3.3.7 Electron microscopy

For scanning electron microscopy (SEM), freshly dissected midgut glands were dehydrated in a series of ethanol, and then subjected to critical-point drying with CO₂. The dried samples were mounted on aluminum stubs with double-sided adhesive tape. The samples were coated with gold in a sputter coater (SCD 050, Zeiss) and observed under a LEO 420 scanning electron microscope (LEO Electron Microscopy Ltd., England).

For transmission electron microscopy (TEM), freshly dissected midgut glands were immediately fixed in a drop of 3.5% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) for 30 min. To ensure rapid penetration of fixative into the lumen, holes were punched into the hepatopancreas with a sterile needle. They were then fixed in the same buffer for 14 h at 4°C. After washing with 0.075 M cacodylate buffer (pH 7.4) for 30 min, the tissue was postfixed with 1% OsO₄ in 0.05 M cacodylate buffer for 2 h at 4°C. After an additional washing for 30 min, the hepatopancreas were dehydrated in a graded ethanol series at room temperature and embedded in Agar 100 resin (Agar Scientific Ltd., England). Ultra-thin sections (60 nm) were contrasted with 2.5% uranyl acetate and lead citrate (Reynolds, 1963) and analyzed using a Philips CM 10 electron microscope.

3.3.8 Nucleotide sequence accession numbers

Nucleotide sequence accession numbers of the 16S rRNA genes cloned in this study are AY500249 (Clone 48) and AY500250 (Clone 59).

3.4 Results

3.4.1 Density and morphology

DAPI staining and whole-cell hybridization of homogenates with fluorescence-labeled oligonucleotide probe EUB338 revealed a dense bacterial colonization in each of the hepatopancreatic ceca of *P. scaber*, ranging from 2.2×10^6 to 1.4×10^8 cells per animal ($n = 15$), and equaling a cell density of about 2×10^6 to 1×10^8 cells per mg dry mass.

The cells had a spherical shape and fell into two size classes with diameters of 0.2–0.3 μm ("small spheres") and 0.5–0.8 μm ("large spheres") (Fig. 1). Most specimens (12 out of 15) harbored both small and large spheres, whereas a few had either large or small spheres.

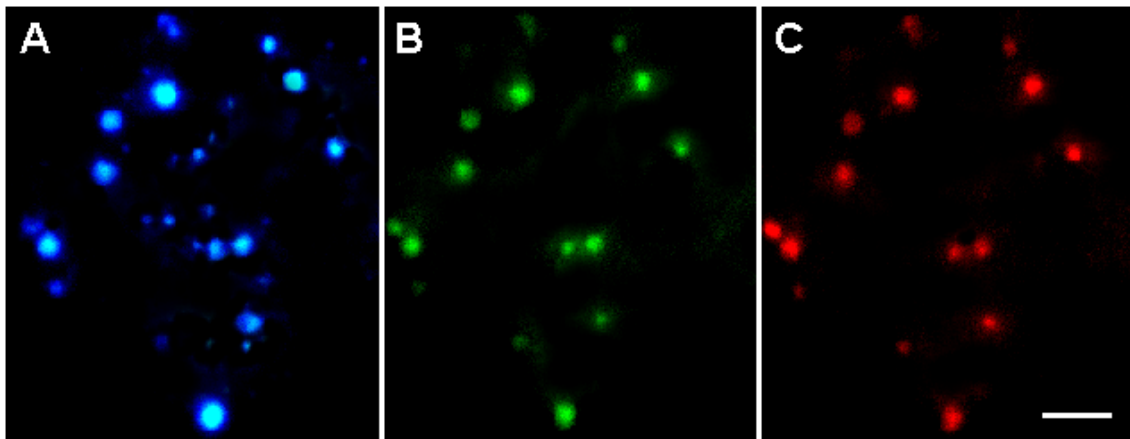


Figure 1. Symbiotic bacteria in a midgut gland homogenate of *Porcellio scaber*. The three epifluorescence photomicrographs show the same microscopic field of a preparation (A) stained with DAPI, and double-hybridized with oligonucleotide probes specific for (B) *Bacteria* (EUB338) and (C) the clones obtained in this study (PsSym352). Hybridization signals of small sphere-shaped symbiotic bacteria faded rapidly during the exposure. Scale bar, 2.5 μm .

3.4.2 Cloning and sequencing

To identify the hepatopancreatic symbiotic bacteria, DNA was extracted from isolated midgut glands, and bacterial 16S rRNA genes were amplified using universal bacterial primers and cloned in *E. coli*. The screened positive clones digested with *MspI*, *AluI*, or *RsaI* endonucleases resulted in 2 different RFLP groups, comprising 12 and 6 clones, respectively, which differed only slightly in their restriction patterns with each of the three enzymes. Sequencing of two representative clones from each RFLP group yielded almost-complete 16S rRNA genes (1517 bp for clone 48 and 1515 bp for clone 59) with virtually identical sequence (99.8 % similarity), which indicated the possibility that two closely related bacterial lineages are present within the hepatopancreas of *P. scaber*.

However, denaturing-gradient gel electrophoresis (DGGE) of 16S rRNA gene fragments amplified by PCR from hepatopancreatic DNA extracts using *Bacteria*-specific primers resulted in electropherograms that contained only a single band, which had the same position in the gel as that of representative clone 48 (Fig. 2). The PCR product of the closely related clone 59, representing the second, less-frequent ribotype in the clone library, had a slightly different melting point; a corresponding band was not present in the hepatopancreatic sample.

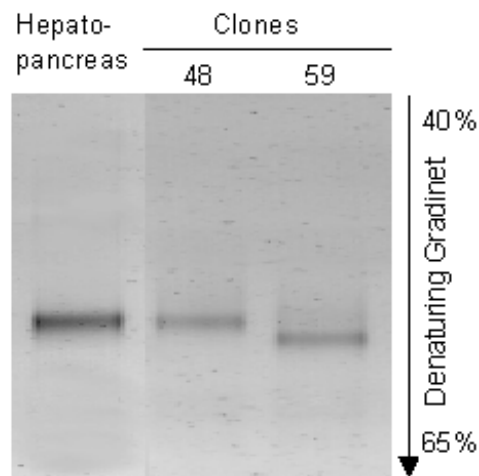


Figure 2. DGGE profiles (negative images) of PCR amplicons obtained from the DNA extracted from the hepatopancreas of *Porcellio scaber* and from two representative clones from the clone library.

3.4.3 Phylogenetic analysis.

BLAST searches gave only low similarities of the hepatopancreatic clones to other bacteria in public databases, but indicated an affiliation with the bacterial class of *Mollicutes*. A detailed phylogenetic analysis based on an extensive dataset comprising all major taxa of *Mollicutes* revealed that the clones are a novel lineage, representing a sister group of the *Spiroplasma* group and *Mycoplasma pneumoniae* group (<82% sequence identity), which contain parasites and pathogens of other animals (Fig. 3). The early branching point at the base of the *Mycoplasmatales* was confirmed by all phylogenetic analyses, using neighbor-joining, maximum-parsimony or maximum-likelihood algorithms, and was also supported by bootstrap analysis (Fig. 3).

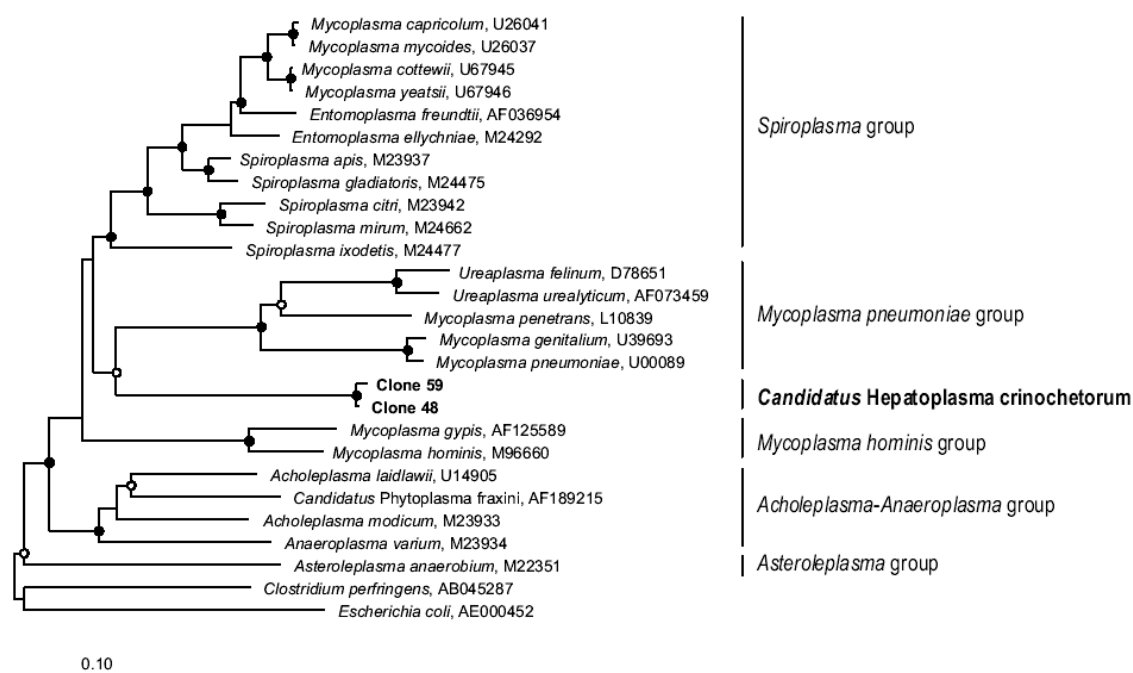


Figure 3. Phylogenetic tree (16S rRNA gene sequences; maximum-likelihood method) showing the evolutionary relationship of the hepatopancreatic symbionts of *Porcellio scaber* (shown in bold) to the selected species from the main phylogenetic clades among the *Mollicutes* (marked with vertical lines). The tree was rooted using *Escherichia coli* and *Clostridium perfringens* as outgroup. Nodes with bootstrap values (DNAPARS, 1,000 replicates) of >99% (●) and >50% (○) are marked. All marked nodes were present also in all other phylogenetic analyses using maximum-parsimony and neighbor-joining algorithms (not shown). Bar represents 10 substitutions per 100 nucleotides.

3.4.4 In situ identification and localization

To exclude the possibility that the apparent homogeneity of the symbiont population was due to PCR bias and to confirm that the clones truly originated from the symbiotic bacteria in the hepatopancreas of *P. scaber* and not from contamination, midgut gland homogenates were subjected to whole-cell hybridization with fluorescence-labeled oligonucleotide probes. In homogenates from 15 different individuals, all DAPI-stained cells (Fig. 1A) hybridized with a *Bacteria*-specific probe (Fig. 1B) and with the group-specific probe PsSym352 (Fig. 1C). The weak hybridization signal of the small spheres

bleached rapidly and is difficult to detect on the image in Fig. 1. Spherical bacterial cells hybridizing with the group-specific probe PsSym352 were also detected in the hepatopancreatic homogenates of other crinochete isopods (*Alloniscus perconvexus*, *Philoscia muscorum*, *Oniscus asellus*, *Trachelipus rathkii*, and *Armadillidium vulgare*). Detailed results will be published in the context of a comparative study on the symbionts in the hepatopancreas of different isopods.

In situ hybridization of thin sections of midgut glands revealed that the symbiotic bacteria were not spread over the lumen of the hepatopancreatic ceca, but were mostly associated with the wall, which is formed by a monolayer of endothelial cells (Fig. 4A). Tangential sections substantiated that the bacteria were not located within the cytoplasm, but colonized the surfaces of the epithelium (Fig. 4B). The same results were obtained with thin sections from specimen harboring only small spheres (not shown), which supports the conclusion that the small spheres and the large spheres represent a homogeneous population rather than two very closely related lineages.

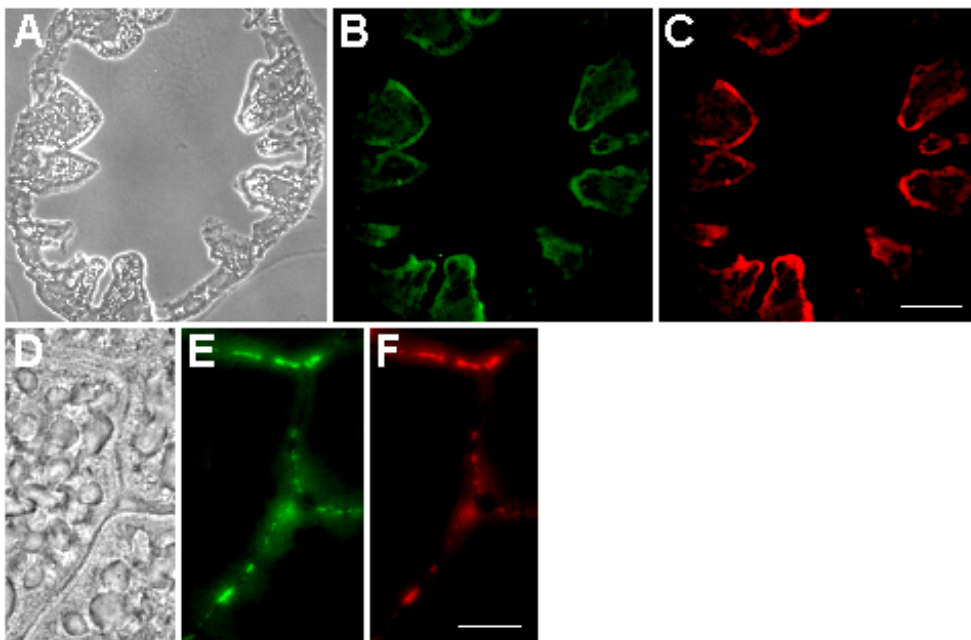


Figure 4. Thin sections of the midgut glands of *Porcellio scaber*, hybridized with fluorescently labelled oligonucleotide probes. The photomicrographs show the same microscopic field of a transverse section (A–C) and a tangential section (D–F), viewed with phase-contrast (A, D) or epifluorescence microscopy, using filter sets for (B, E)

probe EUB338, specific for *Bacteria*, and (C, F) probe PsSym352, a group-specific probe. Scale bars: 100 μm (A–C); 10 μm (D–F).

3.4.5 Morphology and ultrastructure

Both TEM and SEM revealed an intimate association of the symbionts with the epithelial surfaces of the hepatopancreas. Every cecum investigated contained a dense population of bacteria located close to the endothelial cell surface, often occupying the grooves between the protruding epithelial cells (Fig. 5A and Fig. 6A). Bacterial cells were spherical in shape, and large reticulate cells and small cells with dense cytoplasm could be distinguished (Fig. 5A). No cell wall was visible, and cells were surrounded by a single unit membrane (Fig. 5B). Flagella were not observed, but the large cells frequently had stalk-like appendages at one (Fig. 5C,E- and Fig. 6B) or both cell poles (Fig. 5D). The stalks were cytoplasmic protrusions surrounded by a cell membrane (Fig. 5C), had a diameter of 50–100 nm, and varied in length (0.3–1.1 μm). In some cases, the stalks appeared to be involved in budding (Fig. 5F), with the small cells possibly representing the result of an unequal cell division. Often, stalks were in contact with the microvillous brush border and inserted into the space between the microvilli (Fig. 5E).

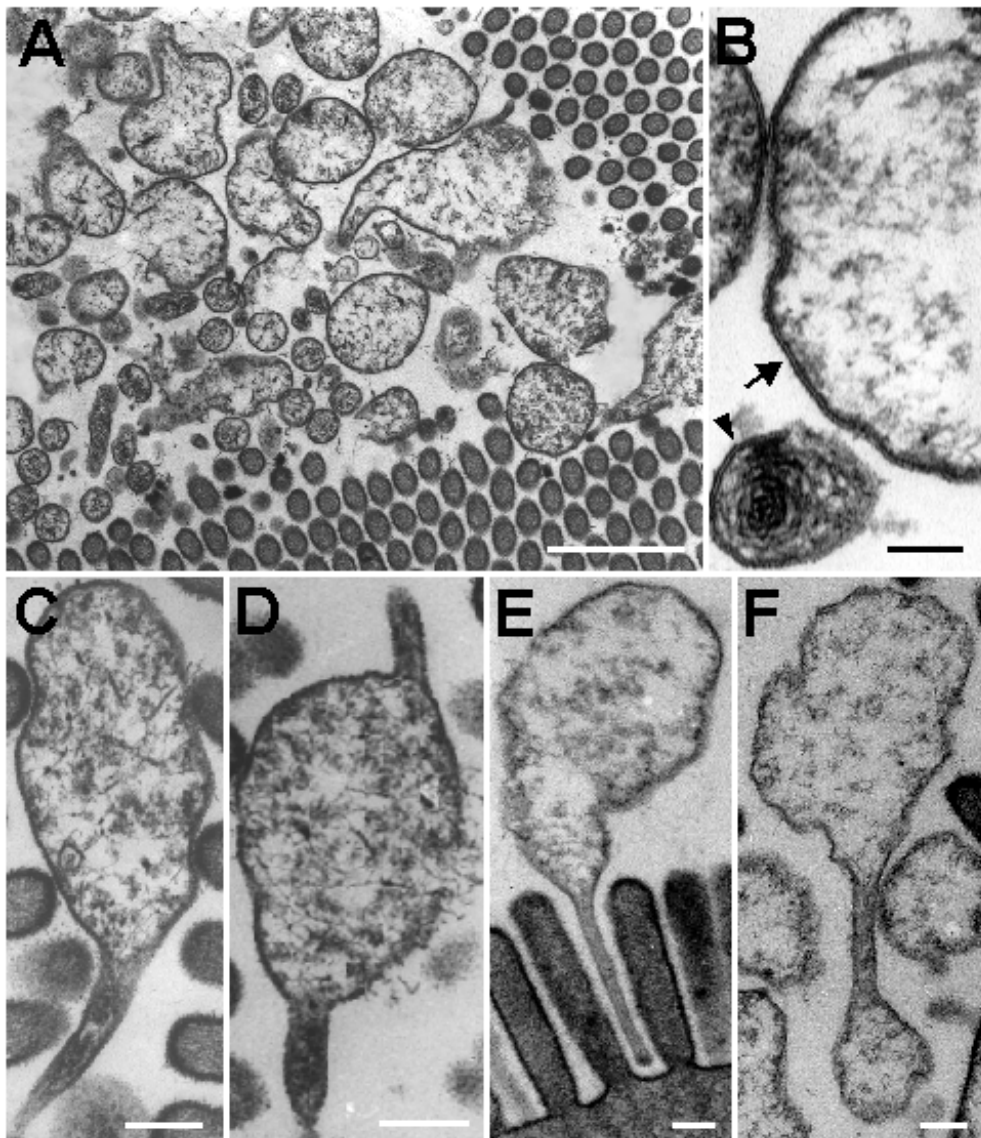


Figure 5. Transmission electron micrographs of bacterial symbionts in the midgut glands of *Porcellio scaber*, showing (A) large and small sphere-shaped cells in close association with the microvilli; (B) both large and small cells lacking a cell wall and bounded by a single unit membrane, marked with an arrow (large cell) and an arrowhead (small cell), respectively; (C) the frequently observed stalk on one cell pole, (D) on both cell poles, and (E) inserted into the gaps between the microvilli of the brush border; and (F) cell division by budding from the stalks. Scale bars: 1 μm (A); 0.1 μm (B); 0.2 μm (C–F).

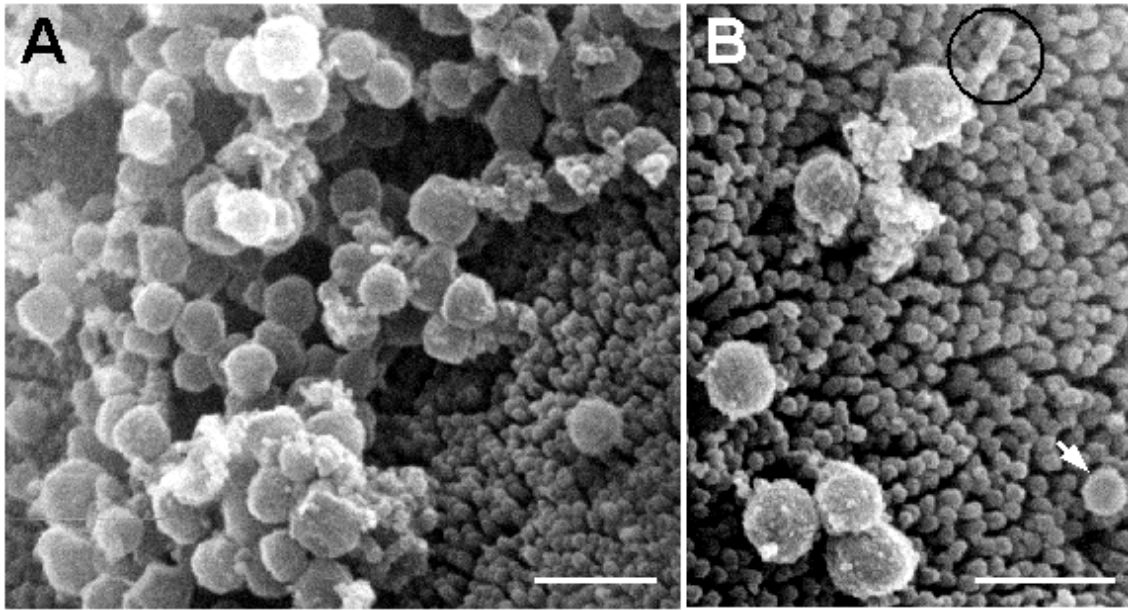


Figure 6. Scanning electron micrographs of bacterial symbionts in the midgut glands of *Porcellio scaber*, showing (A) uniform spherical cells in close association with the microvilli of the brush border, and (B) a large sphere with a stalk (marked with a circle) and a small sphere (marked with an arrow). Scale bars: 1 μm in (A) and (B).

3.5 Discussion

3.5.1 Phylogeny and evolution of *Mycoplasma*-like symbionts

Presently, the *Mollicutes* are considered to consist of five separate phylogenetic clades: the *Asteroleplasma*, the *Acholeplasma*–*Anaeroplasm*a group, the *Mycoplasma hominis* group, the *Mycoplasma pneumoniae* group, and the *Spiroplasma* group (Razin, 2000). All phylogenetic analyses supported that the hepatopancreatic symbionts of *P. scaber* represent a sixth lineage among the *Mollicutes*.

Although *Mollicutes* are widespread commensals or pathogens of humans, mammals, reptiles, fish, plants, and also of other arthropods (Razin *et al.*, 1998), little is known about their occurrence in crustaceans. Apart from a report of a pathogenic mollicute-like bacterium occurring intracellularly in the hepatopancreatic epithelium of shrimps (*Penaeus vannamei*) (Krol *et al.*, 1991), this is the first study demonstrating the

colonization of the hepatopancreatic lumen of a crustacean host by extracellular *Mycoplasma*-like symbionts.

3.5.2 Symbiotic association

Many *Mollicutes* cause only mild and chronic infections, rarely harming their host markedly, or even killing it (Razin *et al.*, 1998), which is considered indicative of an evolutionary advancement towards mutualistic symbiosis. Hence, it is conceivable that also the *Mycoplasma*-like symbionts of *P. scaber* are either benign pathogens, commensals, or mutualistic symbionts. The benefit for the host is difficult to predict, but the presence of the symbionts might diminish the risk of infection of the hepatopancreas by more harmful parasitic or pathogenic microorganisms. Thus, harboring hepatopancreatic symbionts might have enabled isopods to colonize new habitats and extend their geographic distribution. Previous work, based on antibiotic treatment and analysis of the activity of cellulases and phenol oxidases, had led to the hypothesis that symbiotic bacteria in midgut glands might facilitate the digestion of terrestrial food sources (leaf litter) (Zimmer and Topp, 1998b; Zimmer, 1999). The present results render this less likely since there is to date no evidence of degradation of cellulose among the *Mollicutes*.

3.5.3 Stalk function

A flask- or club-like cell shape with a protruding tip or bleb structure has been observed among pathogenic mycoplasmas (Kirchhoff *et al.*, 1984; Razin, 1985), and there is evidence for the presence of cytoskeletal elements in *Mycoplasma pneumoniae* (Hegermann *et al.*, 2002). While the tip-like structures of *Mycoplasma penetrans* and *M. genitalium* are involved in the invasion of host cells (Jensen *et al.*, 1994; Lo *et al.*, 1993), the morphologically different stalks of the *Mycoplasma*-like symbionts of *P. scaber* appear to serve merely in the adhesion to the host endothelium. An analogous function has been proposed also for the stalks of the candidate species 'Hepaticola porcellionum', a *Rickettsia*-like symbiont colonizing the hepatopancreas in other populations of *P. scaber* (Wang *et al.*, 2004). The occurrence of stalk-like structures that insert between the microvilli of the epithelial brush border is rather unusual among the bacterial symbionts of invertebrates, and may be a case of convergent evolution,

driven by the same functional necessities. Whether the latter lies, e.g., in the prevention of a complete washout of the symbionts into the digestive tract together with the hepatopancreatic secretions, or in the facilitation of nutrient exchange with the host epithelium, remains open to speculation and further study.

3.5.4 Small and large spheres

The results of the sequencing analysis document the presence of two closely related 16S rRNA genes in the DNA extracted from the hepatopancreas of *P. scaber*. Coincidentally, also two types of bacterial cells – small and large spheres – were observed to colonize the hepatopancreatic lumen. Both of them hybridized with the sequence-specific probe PsSym352 and possessed the same ultrastructural features. The probe did not allow to differentiate between the two clone groups, and the fact that the DGGE analysis showed only a single band may be explained by PCR bias. However, it is presently not possible to decide whether there are two separate, albeit closely related lineages of symbionts or only a single, morphologically variable strain with two slightly divergent copies of the 16S rRNA gene.

As the symbionts in the hepatopancreas of *P. scaber* share <82% sequence identity with the 16S rRNA genes of their closest relatives in the *Spiroplasma* group and *Mycoplasma pneumoniae* group of the *Mollicutes*, they cannot be assigned to any recognized taxon within this class. Following the recommendation of Stackebrandt et al. (2002), who encouraged the use of the *Candidatus* concept for well-characterized but as-yet uncultured organisms (Stackebrandt *et al.*, 2002), provisional classification of the symbionts as '*Candidatus* Hepatoplasma crinochetorum' is proposed.

3.5.5 Description of '*Candidatus* Hepatoplasma crinochetorum'

Hepatoplasma crinochetorum (he.pa.to.plas'ma. Gr. n. *hepar* liver, Gr. neutr. n. *plasma* something formed or moulded, N.L. neutr. n. *hepatoplasma* something from the liver (hepatopancreas); cri.no.che.to'rum. N.L. gen. pl. of isopods from the taxon *Crinocheta*).

The short description is as follows: Spherical cells (diameter 0.5–0.8 µm); without cell wall and outer membrane; forms stalk-like cytoplasmic appendages at one or two cell poles that attach to host epithelia or are involved in budding. Basis of assignment: 16S rDNA sequences (accession numbers AY500249 and AY500250), hybridization with 16S-rRNA-targeted oligonucleotide probe (5'-GTG AAA AAT TCC CTA CTG CTG-3'). Symbiont of *Porcellio scaber* and other terrestrial isopods of the taxon Crinocheta, located exclusively in the lumen of the hepatopancreas; so far uncultivated.

3.6 References

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4 Symbiotic bacteria in the midgut glands of isopods (Crustacea: Isopoda): phylogeny and evolution

4.1 Abstract

Terrestrial isopods, originating from marine ancestors, harbour uncultivated bacterial symbionts in their midgut glands (hepatopancreas) that may have facilitated the evolutionary shift to utilising leaf litter as food source during the course of colonising terrestrial habitats. In a first molecular-biological approach to understand the evolutionary background of this symbiosis, we screened isopods from marine, semi-terrestrial and terrestrial habitats for bacterial symbionts in their midgut glands by means of 16S rRNA gene sequencing and fluorescence *in situ* hybridisation with probes specific for the small subunit of ribosomal RNA of different bacterial genotypes. DAPI-staining, EUB338-specific hybridisation, and PCR-amplification of eubacterial DNA revealed that there are no hepatopancreatic bacteria in the marine isopod *Idotea balthica*, while high numbers of nearly uniform morphotypes of bacteria were detected in semi-terrestrial (*Ligia oceanica*) and terrestrial isopods (*Oniscus asellus*), respectively. Phylogenetic analyses demonstrated that the symbiotic bacteria of different isopod species, inhabiting semi-terrestrial vs. terrestrial habitats, belong to bacterial taxa as different as γ -Proteobacteria (*Pseudomonas* [similarity >99%] in *L. oceanica*), and the class Mollicutes within the phylum Firmicutes of the Low G+C gram-positive bacteria (*Mycoplasma*-like symbionts [similarity <81%] in *O. asellus*). Fluorescence-labelled oligonucleotide *in situ* hybridisation (FISH) confirmed that the cloned 16S rRNA gene sequences obtained from isopod hepatopancreas originated from the symbionts. The *Mycoplasma*-like symbiont appears to be closely related to *Candidatus* Hepatoplasma crinochetorum, a symbiont of another terrestrial isopod, *Porcellio scaber*. We also detected a *Rickettsia*-like (α -Proteobacteria) symbiont in the hepatopancreas of *O. asellus*, that appears to be closely related to *Candidatus*

Hepaticola procellionum, known from *P. scaber*. Both the low diversity of hepatopancreatic bacteria in single isopod species and the remarkable differences between semi-terrestrial and terrestrial isopod species suggest specific host-symbiont relationships as well as independent acquisition of hepatopancreatic symbionts in different phylogenetic lines of isopods or an evolutionary exchange during the process of isopod terrestrialization. The symbionts' phylogenetic relationship to pathogens indicates a parasitic origin of the recent symbiosis and, further, may shed light on the mechanisms of isopod infection by specific symbiotic bacteria.

4.2 Introduction

It has since long been known that numerous animals of different taxonomic affiliations host microbial symbionts that contribute rare nutrients that the hosts themselves can not synthesise (Buchner, 1965; Douglas, 1998; Moran and Baumann, 2000); in certain invertebrates, symbiotic microorganisms seem to play a crucial role in digestion (Breznak and Brune, 1994; Breznak, 2000; Brune and Friedrich, 2000). However, most bacterial symbionts have not yet been cultured (Amann *et al.*, 1995), which hampers the study of their symbiotic association with, and the benefits for, their hosts.

Isopods (Crustacea: Isopoda) originated in marine habitats but successfully colonised terrestrial habitats. It is commonly accepted that the terrestrial suborder Oniscidea shares a common ancestor with the marine suborders Valvifera and Sphaeromatidea (Zimmer, 2002). Symbiotic bacteria have been described in the digestive and absorptive midgut glands (hepatopancreas) of the terrestrial isopods *Porcellio dilatatus* (Donadey and Besse, 1972), *Porcellio scaber* (Hames and Hopkin, 1989; Wang *et al.*, 2004a; Wang *et al.*, 2004b; Wood and Griffith, 1988; Zimmer and Topp, 1998b; Zimmer, 1999), *Oniscus asellus* (Hames and Hopkin, 1989; Hopkin and Martin, 1982; Wood and Griffith, 1988) and *Ligia pallasii* (Zimmer *et al.*, 2001). One of the most interesting aspects of these symbiotic bacteria concerns their proposed contribution to digestive processes, e.g., the hydrolysis of cellulose (Zimmer and Topp, 1998b; Zimmer *et al.*, 2002) and the oxidation of phenolics (Zimmer, 1999; Zimmer *et al.*, 2002) and lignins (Zimmer and Topp, 1998a; Zimmer *et al.*, 2002). Thus, hepatopancreatic bacteria may have facilitated the evolutionary colonisation of terrestrial habitats by Oniscidea and their utilisation of terrestrial food sources (Zimmer and Topp, 1998b; Zimmer *et al.*, 2002; Zimmer and Bartholmé, 2003). Hepatopancreatic bacteria may either have been acquired simultaneously with numerous adaptations that allowed colonisation of land, or the acquisition of hepatopancreatic bacteria may have been a pre-disposition to the colonisation of land. In the former case, these bacteria would be lacking from marine isopods (e.g., Valvifera, Sphaeromatidea); in the latter case, hepatopancreatic bacteria would be present in marine species (Zimmer *et al.*, 2002). Analysing hepatopancreatic bacteria of isopods from different habitats could therefore be valuable in shedding light

on both the importance of symbionts in digestion, and on the colonisation of land by marine arthropods.

Due to the failure to isolate and cultivate hepatopancreatic bacteria *in vitro* (Wood and Griffith, 1988; Zimmer and Topp, 1998b), little is known about their physiological characteristics and taxonomic affiliation. In the present study, PCR-based methods, hybridisation techniques, and DGGE are employed to investigate the phylogenetic affiliation of symbiotic bacteria in the hepatopancreas of selected isopod species from diverse habitats (marine, semi-terrestrial, terrestrial) in a first attempt to understand the evolutionary background of the host-symbiont relationship, and reveal insights into the symbiotic association between symbionts and their isopod hosts.

4.3 Material and Methods

4.3.1 Collection and culture of animals.

Isopods used in this study are listed in Table 1. *Idotea balthica* (Valvifera: Idoteidae) were collected from seaweeds at the Falckenstein beach near Kiel, Germany, kept in plastic containers (30 cm × 20 cm × 20 cm) filled with artificial seawater (0.8 % [wt/vol] sodium chloride), and fed on brown algae (*Fucus* spp.). *Ligia oceanica* (Oniscidea: Ligiidae) were collected beneath rocks at the Falckenstein lighthouse near Kiel, kept in plastic containers (60 cm × 44 cm × 12 cm) with a bottom of 1 cm wet sand, and fed on brown algae (*Fucus* spp.). *Oniscus asellus* were collected by hand beneath decaying wood in the botanical garden of the Christian-Albrechts-Universität of Kiel, kept in plastic containers (19 cm × 19 cm × 18 cm) with a bottom of 1 cm moist plaster, and offered decaying leaf litter as food.

TABLE 1. Isopods used in this study

Species	Sex ^a	Habitat	Food	Average body size (mm)
<i>Idotea balthica</i> (Idoteidae)	F,M	Marine	Brown algae	17
<i>Ligia oceanica</i> (Ligiidae)	F,M	Semi-terrestrial (Intertidal)	Brown algae	35
<i>Oniscus asellus</i> (Oniscidae)	F,M	Terrestrial	Decaying leaf litter	13

^aF: female; M: male.

4.3.2 Separation and preparation of hepatopancreas

Adult isopods, both males and females, were washed 5 times in autoclaved deionised water then surface-sterilised with 70% ethanol and UV light ($\lambda = 254$ nm) for 1 and 5 min, respectively. After blotting their bodies dry, the hepatopancreas were dissected. For total DNA extraction, hepatopancreas were stored in autoclaved reaction tubes filled with 1 ml acetone (Fukatsu, 1999). For fluorescence *in situ* hybridisation (FISH), they were placed in autoclaved reaction tubes filled with 0.5 ml phosphate-buffered saline (PBS, composed of 0.13 M NaCl, 7 mM Na₂HPO₄, and 3 mM NaH₂PO₄, pH 7.4).

4.3.3 Total DNA extraction

Total DNA of the hepatopancreas (5 individuals of *I. balthica*, and 5 individuals of *O. asellus*) was extracted through bead-beating (Friedrich *et al.*, 2001). Proteins were removed by using NH₄COOCH₃, and DNA was recovered in isopropanol precipitation, followed by further polyvinylpyrrolidone spin column purification according to the protocol by Friedrich *et al.* (Friedrich *et al.*, 2001). For *L. oceanica* (1 individual), cells were lysed through bead-beating. Proteinase K was added at 3 mg/ml and the mixture was incubated at 37°C for 45 min, DNA was purified twice with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1 [vol/vol/vol]), precipitated with 0.7 volume of isopropanol, then rinsed in 70% ethanol (−20°C), and resuspended in PCR water (Sigma). All DNA samples were stored at −20°C until analysis.

4.3.4 PCR

All primers applied in this study are listed in Table 2. The GC clamp for DGGE was attached at the 5'-end of the eubacteria reverse primer S-D-Bact-0907-a-A-15. For *I. balthica* and *O. asellus*, PCR amplification of bacterial 16S rRNA genes by using the eubacteria forward primer S-D-Bact-0007-a-S-21/reverse primer S-D-Bact-1492-a-A-22 was carried out as follows: 1 µl of sample DNA, 5 µl of 10× PCR buffer, 50 µM of dNTP mixture, 0.5 µM of appropriate primer, and 1 U of thermostable *Taq(E)* polymerase (Peqlab, Germany) were used in a 0.2 ml PCR tube (Biozym, Germany). The total volume was adjusted to 50 µl with PCR water (Sigma). Thirty cycles of amplification were performed with a Thermal Cycler (Eppendorf) starting the program

with a denaturing step of 3 min at 94°C followed by 29 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, and a final extension step at 72°C for 7 min. For *L. oceanica*, PCR steps were performed according to the FailSafe™ PCR premix selection kit (Biozym, Germany); otherwise the same technique was applied as mentioned above.

PCR for DGGE using the eubacterial forward primer S-D-Bact-0515-a-S-19 and reverse primer S-D-Bact-0907-a-A-15 with GC clamp was carried out as follows. Except for adding 2 µl of sample DNA, the reaction mastermix was identical to the one described above. Touchdown thermal program was adapted starting with a denaturing step of 94°C for 3 min; 10 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 45 s; the annealing temperature of 60°C was decreased by 1°C every second cycle; 20 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 45 s and a final extension step at 72°C for 5 min.

Vector PCR for detecting the correct insert of 16S rRNA genes was employed by using M13 reverse and M13 forward primers with the protocol described for the first PCR reaction except for using 1 U of thermostable *Taq* polymerase (MBI, Germany) and the appropriate buffer. The total volume was adjusted to 50 µl with PCR water (Sigma). 27 cycles of amplification were performed with a Thermal Cycler (Eppendorf) as follows: 94°C for 3 min, 55°C for 30 s, 72°C for 1 min (One cycle); 94°C for 30 s, 55°C for 30 s, 72°C for 1 min (26 cycles); followed by a final incubation at 72°C for 4 min.

PCR for 16S rRNA genes or segments always had DNA directly extracted from termite hindguts (*Reticulitermes flavipes*) as positive control.

All PCR products were analysed by loading 5 µl aliquots on 1.0% (wt/vol) Seakem LE agarose (BioWhittaker Molecular application) gels in 0.5× TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0). After electrophoresis, gels were stained in ethidium bromide solution (0.5 µg/ml) and photographed under UV light with a digital camera.

TABLE 2. Primers used in this study

Primer ^a	Primer sequence from 5' to 3'	Reference or source
S-D-Bact-0007-a-S-21	AGAGTTTGATCCTGGCTCAG	(Weisburg <i>et al.</i> , 1991)
S-D-Bact-1492-a-A-22	TACGG(C/T)TACCTTGTTACGACTT	(Weisburg <i>et al.</i> , 1991)
S-D-Bact-0515-a-S-19	GTGCCAGCAGCCGCGGTAA	(Henckel <i>et al.</i> , 1999)
S-D-Bact-0907-a-A-15	AATTCCTTTGAGTTT	(Henckel <i>et al.</i> , 1999)
GC-clamp	CGCCCGCCGCGCCCCGCGCCCGCCCGCCCGCCCC GCCCGG	
M13F	CAGGAAACAGCTATGAC	Promega
M13R	CGACGTTGTAAAACGACGGCCAGT	Promega

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

4.3.5 Cloning of PCR-amplified 16S rRNA genes and RFLP analysis

PCR products of 16S rRNA genes were purified with the Easy Nucleic Acid Cycle-Pure Kit (Peqlab) and subsequently ligated into the pGEM-T vector cloning system (Promega) as described in the manufacturer's instructions. The ligation product was then transformed into *Escherichia coli* JM 109 provided in the pGEM-T vector cloning kit. Positive ampicillin-resistant transformants were selected through blue-white screening with IPTG (isopropyl- β -D-thiogalactopyranoside) and X-Gal (5-chloro-4-bromo-3-indolyl- β -D-galactopyranoside) as inducer and substrate, respectively. The correct sizes of the inserts were confirmed with PCR by using pGEM-T specific primers M13 forward and M13 reverse. Amplicons of the correct size were subjected to digestion by using the restriction enzymes *MspI*, *AluI* and *RsaI* (MBI Fermentas) as follows: 18 μ l of PCR products, 2 μ l of Y⁺/Tango 10 \times buffer (MBI), and 0.2 μ l (1 unit) of the respective enzyme were added into a sterile 0.2 μ l PCR tube and incubated at 37°C for 15 h. Aliquots of 10 μ l of each digestion were subjected to electrophoresis (1 \times TBE) by using 4% (wt/vol) NuSieve 3:1 agarose (BioWhittaker Molecular applications) gel. Restriction fragment length polymorphism (RFLP) patterns were compared by eye. Representative clones were cultured in 2 ml LB medium containing 100 μ g/ml ampicillin, and shaken at 1,400 rpm at 37°C for 10 h. Plasmids were extracted and

purified according to the Easy Nucleic Acid Plasmid Miniprep Kit 1 instructions (Peqlab) and subjected to DNA sequencing.

4.3.6 Sequencing of cloned 16S rRNA genes

Double-stranded sequencing of the inserts using primers S-D-Bact-0007-a-S-21 and S-D-Bact-1492-a-A-22 as well as S-D-Bact-0515-a-S-19 and S-D-Bact-0907-a-A-15 was performed on an ABI sequencer by GATC (<http://www.gatc.de>).

4.3.7 Sequence assembling and phylogenetic analysis

Sequences were checked and assembled using the DNASTar software (<http://www.dnastar.com>), and the phylogenetic analysis was done using the software implemented in ARB (<http://www.arb-home.de>) (Ludwig *et al.*, 2004). 16S rRNA gene sequences were compared to sequences in public databases using BLAST (Altschul *et al.*, 1997); closely related sequences were retrieved and added to the alignment. Alignments were always manually corrected. For tree reconstruction, highly variable regions of the 16S rRNA gene sequences and sequence positions with possible alignment errors were excluded by using only those positions of the alignment that were identical in at least 50% of all sequences. Only sequences with more than 1,400 nucleotides were used for the alignment. Phylogenetic analysis utilized the maximum-likelihood, maximum-parsimony, and neighbour-joining algorithms as implemented in ARB.

4.3.8 FISH

Dissected and ruptured hepatopancreas, kept in 500 µl of PBS (see above), were sheared with a pipette tip several times, and mixed completely. After sedimentation of the tissue shreds for 5 min, 450 µl of supernatant was carefully transferred to a new sterile 1.5 ml tube, which contained 50 µl of 40% (wt/vol) formaldehyde. The samples were fixed at 4°C for 15 h. The washing step was performed 3 times by adding 500 µl PBS to the pellets after centrifugation at 10,000 × g for 5 min. Finally, the pellets were dissolved by adding 250 µl PBS and 250 µl of 97% (vol/vol) ethanol, and were stored at -21°C for 1–2 weeks until analysis.

Fixed hepatopancreas samples were immobilized on polycarbonate filters (0.2 μm , Fisher Scientific) and dried (Wagner *et al.*, 1993). Fluorescence-labelled probes employed in this study are listed in Table 3. Hybridisation and microscope counts of DAPI (4',6-diamidino-2-phenylindole)-stained and hybridised cells were carried out according to Wagner *et al.* (Wagner *et al.*, 1993). Fluorescence photomicrographs were taken by using a CCD camera attached to the epifluorescence microscope (Zeiss, Germany) and image analysis program (MagnaFire 2.0, U.S.A).

TABLE 3. Probes used in this study

Probes ^a	Probe sequence from 5' to 3'	Target site (rRNA position) ^b	[%] FA ^c	Reference
NONEUB	ACTCCTACGGGAGGCAGC			(Wallner <i>et al.</i> , 1993)
EUB338	GCTGCCTCCCGTAGGAGT	16S (338-355)	0-50	(Amann <i>et al.</i> , 1990)
ALF1B	CGTTCGYTCTGAGCCAG	16S (19-35)	20	(Manz <i>et al.</i> , 1992)
GAM42a	GCCTTCCCACATCGTTT GCCTTCCCACACTTCGTTT	23S (1027-1043)	35	(Manz <i>et al.</i> , 1992)
PSM G440	CCTTCCTCCCAACTT	16S (440-454)	35	(Braun-Howland <i>et al.</i> , 1993)
PsSym352	GTGAAAAATTCCCTACTGCTG	16S (352-373)	0-20	(Wang <i>et al.</i> , 2004a)
PsSym120	AGCCAAATTCACCGTGT	16S (120-138)	20	(Wang <i>et al.</i> , 2004b)

^a 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](http://www.interactiva.de)). NONEUB, negative control probe complementary to EUB338; EUB338, most *Bacteria*; ALF1B, α -Proteobacteria, some δ -Proteobacteria, Spirochaetes; GAM42a, γ -Proteobacteria; PSM G440, *Pseudomonas* spp.; PsSym352, *Candidatus* Hepatoplasma crinochetorum symbionts; PsSym120, *Candidatus* Hepatincola porcellionum symbionts.

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

^c Percent amount of formamide (vol/vol) in the hybridisation buffer.

4.3.9 DGGE

Denaturing gels were prepared by using two 6.5% (wt/vol) acrylamide gel stock solution (37.5:1, acrylamide-N,N'-methylenebisacrylamide in 1 \times Tris-acetate-EDTA [TAE], [containing 40 mM Tris base, 20 mM sodium acetate, and 1 mM EDTA-Na₂]) with 0 and 80% denaturant (100% denaturant contained 7 M urea and 40% [vol/vol] formamide in 1 \times TAE), respectively. Linear 40-80% denaturant gradient was prepared

according to the method of Myers et al. (Myers *et al.*, 1987). After pre-electrophoresis for 30 min, PCR amplicons were loaded onto the gel, which was run at 60°C and 60 V for 15 hours. The gel was stained with ethidium bromide (0.5 µg/ml) for 15 min and photographed under UV light with a digital camera.

4.3.10 Nucleotide sequence accession numbers

The sequences of the 16S rDNA clones obtained from the hepatopancreatic symbionts have been deposited in the GenBank database under accession numbers AY539721-AY539726.

4.4 Results

4.4.1 Morphology and density of symbiotic bacteria

For each specimen, at least 500 DAPI-stained cells from 10-20 microscopic fields were counted. Neither by DAPI-staining nor by whole-cell hybridisation with fluorescence-labelled oligonucleotide probes (EUB338) could we detect any bacteria in the midgut glands of the marine isopod *I. balthica* (Valvifera). In contrast, rod-shaped bacteria were observed in the semi-terrestrial isopod *L. oceanica* (Oniscidea; Table 4 and Fig. 1A), and spherical (Fig. 1B) as well as curved rod-shaped bacteria (Fig. 1C) were detected in the terrestrial isopod *O. asellus* (Oniscidea; Table 4), which morphologically correspond to the symbionts found in the terrestrial isopod species *Porcellio scaber* (Wang *et al.*, 2004a). The symbiont densities (mean values) were significantly different in different species (*I. balthica*, 0; *L. oceanica*, 1.5×10^7 ; *O. asellus*, 2.4×10^8) (Table 4).

TABLE 4. Densities and morphotypes of symbiotic bacteria in different isopod species

Isopod species	Characteristics of symbiotic bacteria		
	Density (per animal)	Morphotype	Size (µm)
<i>I. balthica</i> (n = 30)	–	–	–
<i>L. oceanica</i> (n = 10)	$(0.3 - 6.0) \times 10^7$	Rod	$(1.8-2.5) \times 0.8$
<i>O. asellus</i> (n = 5)	$(0.6 - 9.3) \times 10^8$	Sphere	0.5–0.8 (Diam)
<i>O. asellus</i> (n = 1)	3.5×10^8	Curved rod	$(1.5-3.8) \times 0.5$

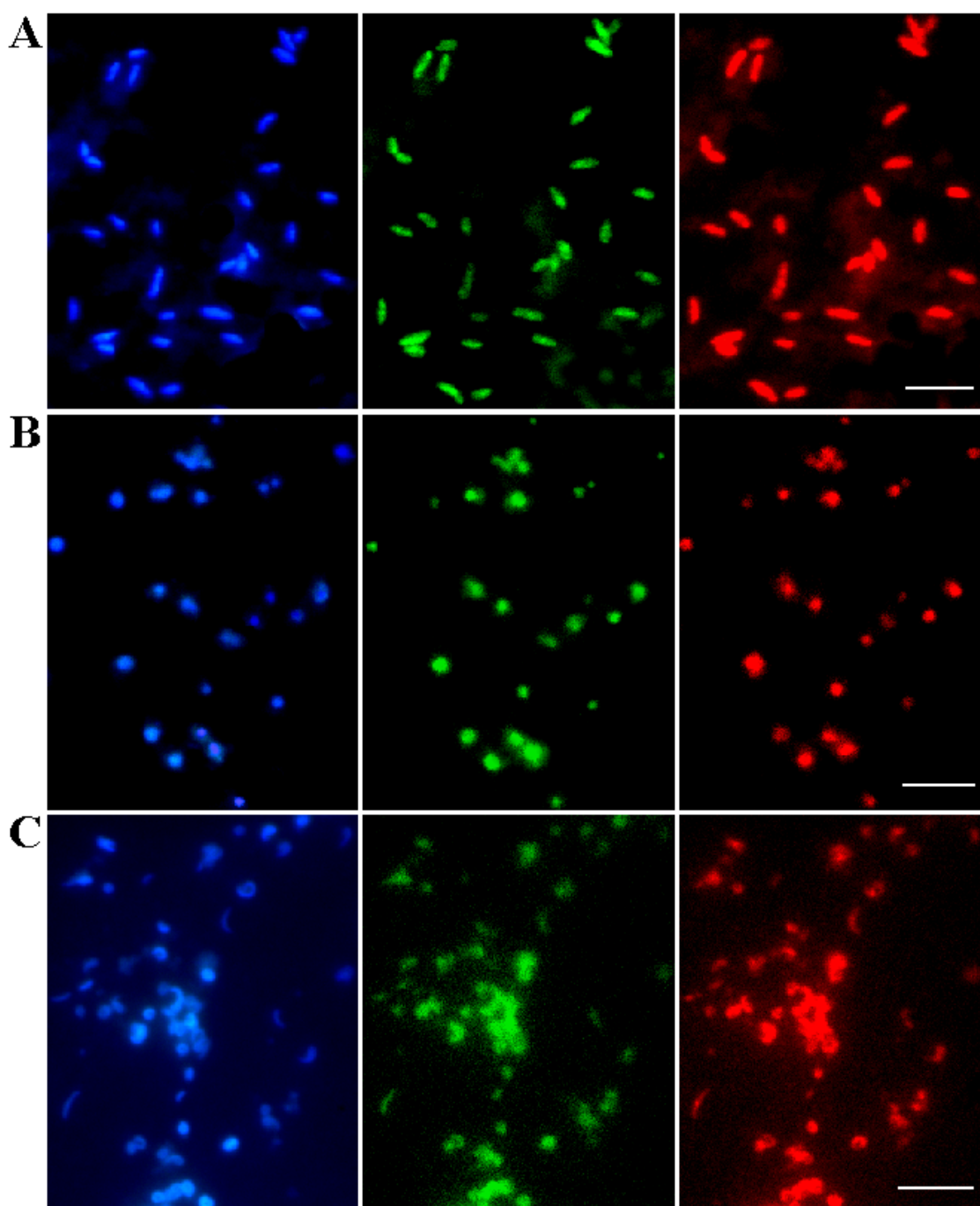


FIG. 1. Epifluorescence microphotographs of symbiotic bacteria in the homogenates of the midgut glands of (A) the semi-terrestrial *Ligia oceanica* and (B, C) the terrestrial *Oniscus asellus* after fluorescence-labelled oligonucleotide probe *in situ* hybridisation. Blue colour, stained with DAPI; Green colour, hybridised with (A) probe GAM42a specific for γ -subclass of the Proteobacteria, (B) probe EUB338 specific for Bacteria, (C) probe ALF1b specific for α -subclass of the Proteobacteria; Red colour, hybridised

with (A) probe PSM G440 specific for the members of the genus *Pseudomonas*, (B) probe PsSym352 specific for the cloned sequences from *O. asellus*, (C) probe PsSym120 specific for *Candidatus Hepatincola porcellionum*. Bar, 5 μm (applies to the same microscopic panels).

4.4.2 PCR amplification, cloning, and RFLP analyses

Data of PCR amplification for 16S rRNA genes, cloning and RFLP analysis are summarised in Table 5. PCR products were successfully amplified from *L. oceanica* and *O. asellus*, using eubacteria primer pairs, however no amplicons were obtained from *I. balthica*, which is consistent with the lack of bacteria in microscopic observations of DAPI-stained samples. The clone groups among both *L. oceanica* and *O. asellus* (RFLP I vs. RFLP III) differed only slightly in their endonuclease restriction patterns with each of the three enzymes *MspI*, *RsaI*, and *AluI*. One representative clone was chosen for sequencing from these sets of RFLP patterns (Table 5).

TABLE 5. PCR amplification, cloning and RFLP analysis of the 16S rRNA genes from symbiotic bacteria of different isopod species

Isopod species	PCR products (kb)	Positive clones ^a	RFLP groups		
			I	II	III
<i>I. balthica</i>	–	–	–	–	–
<i>L. oceanica</i>	~1.5	40 (33) ^b	17 (8) ^c	16 (6)	–
<i>O. asellus</i>	~1.5	28 (12)	6 (2)	4 (12)	2 (13)

^a Ampicillin-resistant and white clones.

^b Number without parenthesis: total number of screened positive transformants; number with parenthesis: number of clones with the correct insert of PCR products.

^c Number without parenthesis: number of clones with identical RFLP profiles; number with parenthesis: selected representative clones for sequencing.

4.4.3 Phylogenetic position of symbiotic bacteria

Assembled base pairs and affiliation of 16S rRNA gene sequences cloned from different isopod species are given in Table 6.

TABLE 6. Sequence analysis results of cloned 16S rRNA genes from hepatopancreatic symbionts in isopod *Ligia oceanica* and *Oniscus asellus*

<i>Isopod</i>	Clone no.	Assembled length (bp)	Sequence affiliation		Most closely related bacterial sequence	% Identity
			Subclass	Order		
<i>L. oceanica</i>	6	1509	γ -Proteobacteria	Pseudomonadales	<i>Pseudomonas</i> sp. NKB1	99.4
	8	649 (5'-)	γ -Proteobacteria	Pseudomonadales	<i>Pseudomonas</i> sp. NKB1	99.2
	8	623 (3'-)	γ -Proteobacteria	Pseudomonadales	<i>Pseudomonas</i> sp. NKB1	99.4
<i>O. asellus</i>	2	1513	Mollicutes	Entomoplasmatales	<i>Spiroplasma diabroticae</i>	79.8
	12	1514	Mollicutes	Entomoplasmatales	<i>Spiroplasma diabroticae</i>	79.6
	13	1517	Mollicutes	Entomoplasmatales	<i>Spiroplasma diabroticae</i>	80.4

Homological comparison showed that clone 6 from *L. oceanica* has a sequence identity of 99% with clone 8, suggesting a close phylogenetic relationship between them. Comparison with the 16S rRNA gene sequences in public databases revealed very high similarities (>99%) with sequences from the bacterial genus *Pseudomonas* (γ -Proteobacteria) for both clones. Both neighbour-joining and maximum-likelihood methods revealed the same tree topology and lineage affiliation of clones 6 and 8 (Fig. 2A).

Nearly full lengths of 16S rDNAs were cloned from *O. asellus* hepatopancreas (Table 6). Clone 2 had a sequence similarity of 99.6% (differed by 9 nucleotides) and 98.1% (differed by 14 nucleotides) with clones 13 and 12, respectively, and the 16S rDNA sequence of clone 12 matched that of clone 13 by 98.6% (differed by 11 nucleotides). These results indicated that the three cloned 16S rRNA genes share close phylogenetic affiliations. The predicted secondary structures for clones 2, 12 and 13 (Figures not shown) share well-complementary stem, suggesting that the retrieved sequences are not due to PCR artifact and encode a functional rRNA. Comparison with the sequences in public databases placed all of them into the bacteria subclass Mollicutes. A detailed analysis revealed only low similarities (<81%) with sequences from the bacteria order Mycoplasmatales and Entomoplasmatales; most closely related bacteria species

(similarity, 79–81%) were found in the genera *Mycoplasma* and *Spiroplasma*. However, these three clones gave high (97.6–98.7%) sequence similarity with the 16S rRNA genes retrieved from the sphere-shaped, *Mycoplasma*-like hepatopancreatic symbionts, harboured by *P. scaber* (Wang *et al.*, 2004a), which indicates that they are intimate lineage members. Phylogenetic trees reconstructed with both neighbour-joining and maximum-likelihood methods revealed that clones 2, 12 and 13 were grouped with the *Mycoplasma*-like symbionts of *P. scaber* together into a single, deeply branched cluster, forming a monophyletic group only distantly related to other lineages of Mollicutes (Fig. 2B).

4.4.4 FISH analysis of the species composition of symbiotic bacteria

In order to exclude contamination and to confirm that the cloned 16S rRNA genes originated from the symbiotic bacteria in the hepatopancreas of isopods, whole-cell hybridisation with fluorescence-labelled oligonucleotide probes was performed. Only the cells which had hybridisation signals stronger than any observed background were counted as positive. For each species, probe NONEUB was employed as the negative control, which never showed any detectable signals of hybridisation in our samples (Figures not shown). All symbiotic bacteria in the hepatopancreas of *L. oceanica* exhibited strong hybridisation signals with probe EUB338 for Bacteria (Figures not shown), GAM42a for γ -Proteobacteria (with BET42a as competitor) (Fig. 1A, green colour), and PSM G440 (no mismatch in target sites of the cloned 16S rRNA sequences) for the members of the genus *Pseudomonas* (Fig. 1A, red colour), which confirmed that the cloned 16S rRNA gene sequences originated from the hepatopancreatic symbionts as well as corroborated the phylogenetic analysis. Sequence-specific Probe PsSym352 was designed to detect the *Mycoplasma*-like hepatopancreatic symbionts in *P. scaber* (Wang *et al.*, 2004a). Sequence analysis revealed that it also completely matches the target sequence of all three cloned 16S rRNA genes from *O. asellus*. Consequently, it was also employed as a specific probe in this study. Our data showed that 100% of DAPI-stained, sphere-shaped cells revealed distinguished hybridisation signals with probe EUB338 (Fig. 1B, green colour) and with the probe PsSym352 (Fig. 1B, red colour), which indicates the cloning work is free of

contamination and supports the close relationship between the *Mycoplasma*-like symbionts found in both *O. asellus* and *P. scaber* as retrieved by phylogenetic analysis. The curved rod-shaped hepatopancreatic bacteria found in *O. asellus* hybridised with Bacteria-specific probe (EUB338) (figures not shown), α -Proteobacteria-specific probe (ALF1B) (Fig. 1C, green colour), and sequence-specific probe (PsSym120) (Wang *et al.*, 2004b) (Fig. 1C, red colour), suggesting that the morphologically identical symbionts found in both *O. asellus* and *P. scaber* belong to the same bacterial lineage (also see below DGGE analysis).

4.4.5 DGGE analysis of the species composition of symbiotic bacteria

To evaluate the species diversity of the symbiotic bacteria in the hepatopancreas of isopod species, DGGE was carried out. In agarose gel, PCR revealed a band at approximately 500-bp for *L. oceanica* and *O. asellus*, but not for *I. balthica* (Figures not shown). Subsequently, the PCR products were loaded onto a denaturing gel and subjected to DGGE. Two bands were detected for *O. asellus*, and one band for *L. oceanica* (Fig. 3), which clearly indicates different hepatopancreatic symbionts in isopod species collected from semi-terrestrial and terrestrial habitats as well as a low symbiont diversity in each isopod species. The DGGE electropherograms of *O. asellus* symbionts were in accordance to that of the *Rickettsia*-like and *Mycoplasma*-like symbionts in *P. scaber*, respectively (Fig. 3), which corroborates the phylogenetic and FISH analyses results (see above), and indicates the close relationship of both the *Mycoplasma*-like and the *Rickettsia*-like symbionts between these two terrestrial isopod species.

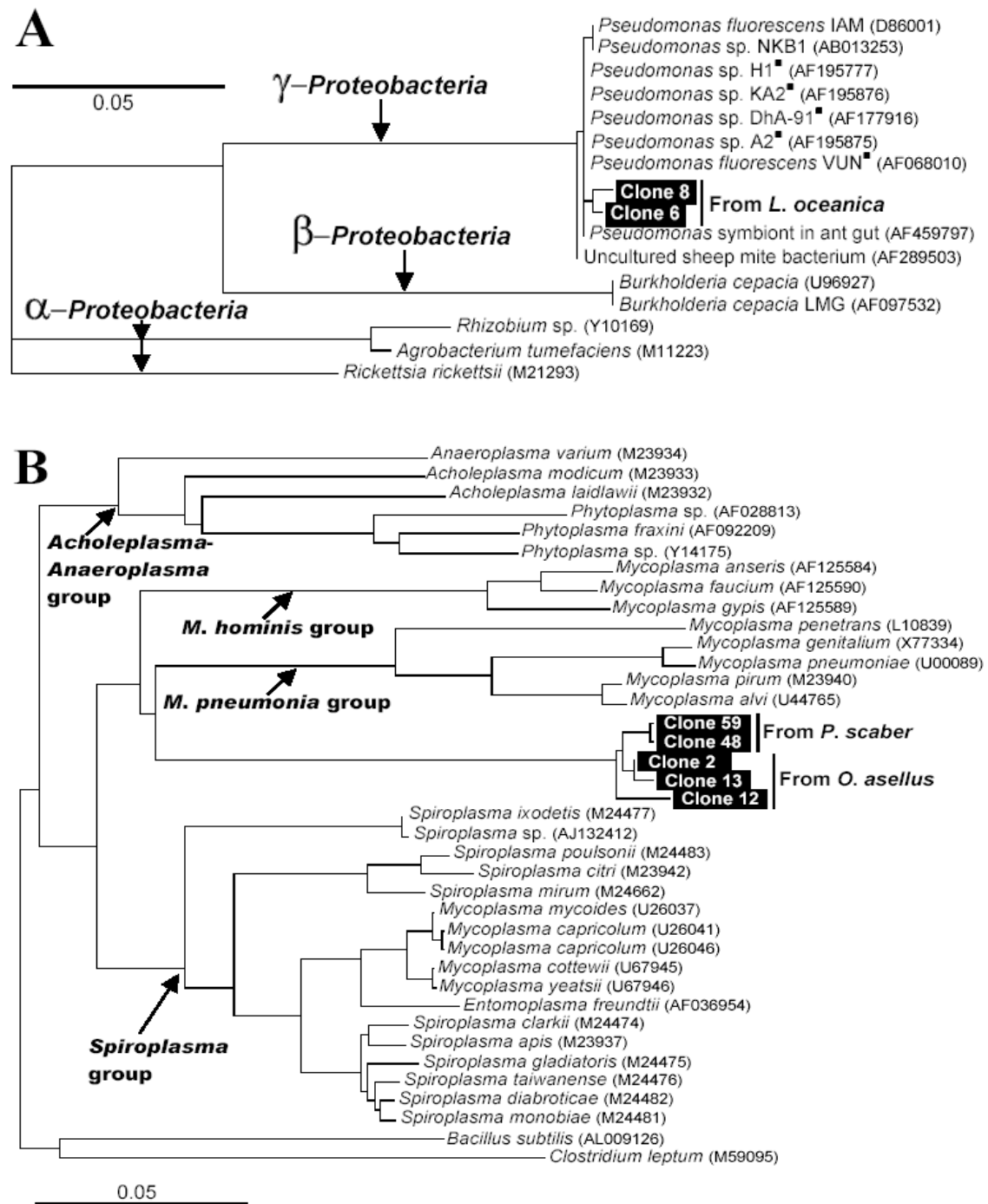


FIG. 2. Phylogenetic tree (maximum likelihood method) showing the relationship of the cloned 16S rRNA genes from the symbiotic bacteria in the midgut glands of the semi-terrestrial *Ligia oceanica* (A) and the terrestrial *Oniscus asellus* (B) to their closest relatives in *Pseudomonas* and reference strains from α - and β -subclass of the Proteobacteria (A), and to their close relatives among the main groups (marked in the figure) of the Mollicutes (B), respectively. Sequences cloned in this study and cloned

from *Porcellio scaber* (Wang *et al.*, 2004a) are presented in white on a black background. Strains marked with (■) in (A) exhibit polycyclic aromatic hydrocarbon-degrading characteristics. GenBank accession number follows the strain name. The sequences of *Rickettsia rickettsii* for (A) and *Bacillus subtilis* and *Clostridium leptum* for (B) were used as outgroup. The bar indicates an estimated sequence divergence of 5%.

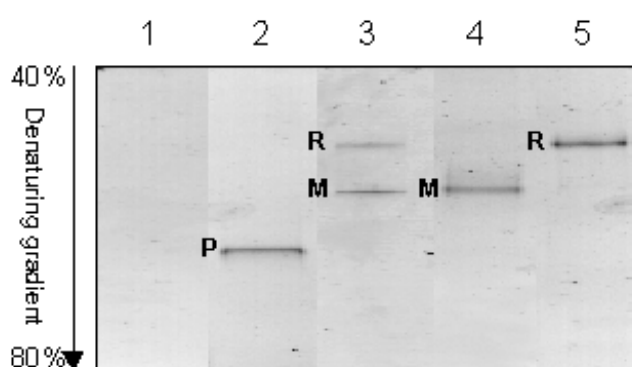


FIG. 3. DGGE fingerprints of hepatopancreatic symbionts colonizing different isopod species from diverse habitats. Lane 1, *I. balthica*; Lane 2, *L. oceanica*; Lane 3, *O. asellus*; Lanes 4 and 5, *P. scaber* (Wang *et al.*, 2004a). Bands marked with (R), (M) and (P) correspond to the *Rickettsia*-like (*Candidatus* Hepatincola porcellionum), the *Mycoplasma*-like (*Candidatus* Hepatoplasma crinochetorum), and *Pseudomonas* symbionts, respectively.

4.5 Discussion

The role of symbiotic bacteria in the midgut glands of isopods has been considered important with respect to the digestive physiology and evolution of isopods (Zimmer and Topp, 1998a; Zimmer *et al.*, 2001). However, precise information on these bacteria is not available until now, except for their morphology mentioned by several authors. In recent reports, we documented unusual, stalk-forming α -Proteobacteria and Mollicutes, representing novel lineages among the bacteria orders Rickettsiales and Mycoplasmatales, respectively, in the hepatopancreas of a terrestrial isopod, *Porcellio scaber* (Wang *et al.*, 2004a; Wang *et al.*, 2004b). Comparative analyses of the phylogenetic positions of these bacterial symbionts with those harboured in different

isopod species may shed light on the origin and evolution of the symbiosis as well as on possible relationships between the symbiotic bacteria and isopod hosts.

4.5.1 Symbiont morphotype and density

Morphotype and cell size of symbionts differed between different isopod species. We found little morphological variability in semi-terrestrial (*L. oceanica*, Oniscidea) and terrestrial (*O. asellus*, Oniscidea) isopods. These interspecific differences in bacterial morphotypes are confirmed by our results of phylogenetic analysis. Notably, several previous reports presented rod-shaped cells in the hepatopancreas of *O. asellus* (Hames and Hopkin, 1989; Hopkin and Martin, 1982; Wood and Griffith, 1988), which are obviously different from the sphere-shaped and curved cells we describe here. This discrepancy may be due to (1) the geographical distribution of the isopods, or (2) the diversity of symbionts in different isopod populations. Detailed investigations on this subject are in progress in our laboratory.

The density of bacterial symbionts in different isopod species was different, too. No bacteria were found in the midgut glands of the marine isopod *I. balthica*, which was corroborated by the PCR analysis for 16S rRNA genes. In coincidence, no bacteria were found in the hepatopancreas of *Idotea wosnesenskii* (Valvifera: Idoteidae) (Zimmer *et al.*, 2001), indicating that at least these two marine species, as well as the intertidal *Gnorimosphaeroma oregonense* (Sphaeromatidea: Sphaeromatidae) (49), do not harbour any hepatopancreas symbionts. According to the currently accepted phylogeny of Isopoda (see Introduction), these species represent the sister taxon of Oniscidea. By contrast, in *L. oceanica*, belonging to a genus that represents a prototypal terrestrial isopod with respect to adaptations to the terrestrial habitat (Carefoot and Taylor, 1995; Schmalzfuss, 1978), 1.5×10^7 cells per animal were detected, which coincides with bacterial numbers found in *Ligia pallasii* (Oniscidea: Ligiidae) (Zimmer *et al.*, 2001). These observations lead us to conclude that (1) the existence of hepatopancreatic symbionts is closely related to the terrestrialization of isopods, and (2) hepatopancreatic bacteria were acquired simultaneously with morphological, physiological and behavioural adaptations that allowed colonisation of land (see Introduction) after the phylogenetic separation of Oniscidea and the lineage of Valvifera and Sphaeromatidea.

Assuming the use of hepatopancreatic symbionts in digesting cellulose and phenolics (Zimmer and Topp, 1998a, b; Zimmer, 1999), the variation in the density of symbiotic bacteria (0 [*I. balthica*] $< 2.2 \times 10^5$ [*L. oceanica*] $< 2.2 \times 10^8$ [*O. asellus*] cells/mg dry weight) may reflect one of the adaptations that accompanied the nutritional shift to feeding on terrestrial food sources (see Introduction; (Zimmer and Bartholmé, 2003)).

4.5.2 Phylogenetic analysis and symbiotic association

The closest relative of hepatopancreatic bacteria in the semi-terrestrial isopod *L. oceanica*, *Pseudomonas* sp. NKB1, is a free-living bacterium found in deep-sea sediments (Li *et al.*, 1999) (Table 6 and Fig. 2A), hinting on symbiotic bacteria initially originating from the marine environment. Considering both that *L. oceanica* inhabits the coastal marine environment and the lack of hepatopancreatic bacteria in marine isopods, this conclusion also suggests an acquisition of bacterial symbionts at an early stage of terrestrialization in a semi-terrestrial (intertidal) environment (see above).

Other close relatives of the *Ligia* symbionts, e.g. *Pseudomonas* sp. KA2 and *Pseudomonas fluorescens* strain VUN (similarity, >98%) (Fig. 2A), are able to degrade polycyclic aromatic hydrocarbons by oxygenase; many other *Pseudomonas* spp. show the capability of degrading diverse aromatic hydrocarbons (<http://bsd.cme.msu.edu/bsd>), and a peroxidase produced by *Pseudomonas paucimobilis* SYK-6 degrades various dimeric lignin compounds (Masai *et al.*, 1991). In addition, cellulase and hemicellulase (xylanase), needed for the degradation of plant cell walls, are produced by *Pseudomonas fluorescens* ssp. *cellulosa* (Hazlewood and Gilbert, 1998). Given that the phylogenetic relationships of closely related bacteria species reflect phenotypic similarity (Moore *et al.*, 1998), the close phylogenetic relationship between these degraders and the symbionts suggests the potential of *Pseudomonas* symbionts in *L. oceanica* to contribute to the digestion of decaying seaweed and encrusting diatoms, and possibly even terrestrial food sources, by the host. Recently, symbiotic bacteria related to *Pseudomonas* were found in the gut of *Tetraponera* ants (van Borm *et al.*, 2002); these nitrogen-recycling endosymbionts have >98% sequence similarity with hepatopancreatic symbionts of *L. oceanica* (Fig. 2A). Although nitrogen is abundant in seaweed tissue, its content is significantly reduced in decaying seaweed, the major food

source of *Ligia* (Indergaard and Minsaas, 1991). Thus, symbiotic interactions with respect to nitrogen fixation alike that between *Tetraponera* ants and their gut symbionts may be important in the semi-terrestrial *L. oceanica*, too, and may have been significant for further steps of terrestrialization.

The closest relatives of *O. asellus* hepatopancreatic symbionts revealed by phylogenetic analysis are the *Mycoplasma*-like bacteria in terrestrial isopod *P. scaber*, which represents a novel clade among Mollicutes as well as a sister group of the *Mycoplasma pneumoniae* group and the *Spiroplasma* group (Fig. 2B). Mycoplasmas are widely distributed as benign pathogens or parasites in humans, many animals (including other arthropods), and plants, exhibiting strict host and tissue specificities (Razin, 1992). However, besides reports of a pathogenic mollicute-like bacterium occurring intracellularly in the hepatopancreatic epithelium of shrimps (*Penaeus vannamei*) (Krol *et al.*, 1991) and a pathogenic *Spiroplasma* bacterium infecting in the brain tissue of crab (*Eriocheir sinensi*) (Wang *et al.*, 2003), mycoplasmas or *Mycoplasma*-like bacteria have not been observed in any isopod species other than *O. asellus* (this study) and *P. scaber* (Wang *et al.*, 2004a). Furthermore, the distinguished pathological symptoms involved in the modification of animal behaviour and the cell and tissue structures of the hepatopancreas accompanying such infections were not observed in our experimental isopods. Hence, hepatopancreatic symbionts in *O. asellus* alike that in *P. scaber* (Wang *et al.*, 2004a) possibly represent a non-pathogenic lineage of mycoplasmas that likely derived from parasites but evolved towards mutualistic symbioses (Price, 1991). The closest relative (albeit distant), the intracellular *Spiroplasma* (Mollicutes) in the two-spot ladybird beetle, *Adalia bipunctata*, takes the role of male-killing, which results in not only directly enhancing their spread through host populations but also, indirectly, contributing to the female host fitness (Hurst *et al.*, 1999). In contrast, *Spiroplasma* symbionts of the pea aphid, *Acyrtosiphon pisum*, closely related to that of the beetle, exhibit negative effects on the host fitness (Fukatsu *et al.*, 2001). Given the low sequence similarity (<80%) to these symbionts, the symbiotic association of *Mycoplasma*-like symbiotic bacteria in terrestrial isopods can be expected to be different and maybe even beneficial to the host.

Intriguingly, the *Rickettsia*-like symbionts found in *P. scaber* (Wang *et al.*, 2004a) also occurred in *O. asellus*. Other crustaceans, e.g., decapods shrimp, could be infected with intra-cellular pathogenic Rickettsiae members (Vogt, 1997), or *Rickettsia*-like bacteria (Krol *et al.*, 1991; Loy *et al.*, 1996) in their hepatopancreatic cells. However, they are distantly related to the *Rickettsia*-like symbionts in the hepatopancreas of isopod hosts. In addition, the high mortality rates in response to such infections were not observed in our experimental isopods. Therefore, although the symbiotic association between the *Rickettsia*-like symbiont and isopod host remain unclear, pathogenic features of the symbiotic bacteria seem to be unlikely.

In contrast to the proposed food-digesting role of hepatopancreatic symbionts, neither cellulase or hemicellulase (xylanase) nor lignin peroxidase have been found in Mollicutes and Rickettsiales thus far (Lynd *et al.*, 2002). Hence, it appears unlikely that these symbionts aid the host in digesting food (leaf litter). However, we hypothesise that hepatopancreatic symbionts are somehow involved in the nutritional physiology of their hosts. Future work is needed to verify this speculation.

4.5.3 Evolution of symbiosis

Corroborating our phylogenetic analysis of the 16S rDNA genes, the DGGE profiles show low intraspecific variability but high interspecific (semi-terrestrial species *vs.* terrestrial species) variability in hepatopancreatic bacteria of different isopod species (Fig. 3), indicating weak co-evolutionary convergence of symbiotic bacteria and isopod hosts on a larger phylogenetic scale, or an evolutionary exchange of symbionts during isopod phylogeny. The evolutionary history of isopods involved changes of habitats from strictly marine to semi-terrestrial and terrestrial, food sources from seaweed to leaf litter, and both physiological and morphological characteristics (Zimmer, 2002). Thus, requirements of different isopod species from these diverse habitats with respect to characteristics of bacterial symbionts are significantly different, possibly resulting in an evolutionary exchange of symbionts as our present results suggest. On the other hand, however, both *O. asellus* and *P. scaber*, truly terrestrial Oniscidea, harbour very closely related hepatopancreatic symbionts, *Mycoplasma*-like and *Rickettsia*-like bacteria, according to the morphology, phylogeny, FISH, and DGGE analysis. This possibly

mirrors the history of the association between hosts and symbionts and suggests a single infection of a common ancestor of these isopod species followed by cospeciation of host and hepatopancreatic symbionts. To further corroborate this conclusion, work regarding a broad survey for the geographical distribution of the symbionts in intra- and inter-species of terrestrial isopods is in progress in our laboratory.

In conclusion, our data indicate that (1) although symbiotic bacteria colonizing semi-terrestrial and terrestrial isopod species belong to different taxa, the occurrence of symbionts is closely related to isopod evolution towards terrestrial land; (2) specific associations of different isopod species with bacterial symbionts reflect their different ecological habitats; (3) symbiotic bacteria in terrestrial isopod species (*P. scaber* and *O. asellus*) possibly underwent an evolutionary exchange or represent independent origins of symbiotic associations in different lines of Oniscidea; (4) communities of hepatopancreatic bacteria in particular isopod species are characterised by low diversity; (5) a parasitic and/or pathogenic origin of hepatopancreatic symbionts and co-evolution of them and their isopod hosts are possible in truly terrestrial isopod species; (6) a symbiotic association involving the digestion of leaf litter was not strongly substantiated, but benefits for the host resulting from physiological characteristics of symbionts are possible, and need further clarification.

4.6 References

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5 Diversity of hepatopancreatic symbionts in the freshwater isopod, *Asellus aquaticus* (Crustacea: Asellota)

5.1 Abstract

Freshwater isopods, *Asellus aquaticus*, harbour uncultivated symbiotic bacteria with high diversity between populations in their midgut glands (hepatopancreas). Phylogenetic affiliations of the symbionts were determined by cloning, sequencing, and comparatively analysing the bacterial 16S rDNA sequences. They are closely associated with members of the bacterial genera *Rhodobacter* (α -Proteobacteria, similarity >95%), *Burkholderia* (β -Proteobacteria, similarity >99%), *Aeromonas* (γ -Proteobacteria, similarity >99%), and *Rickettsiella* (γ -Proteobacteria, similarity >96%), respectively. The γ -Proteobacterial symbiont clustered with *Rickettsiella grylli*, an intracellular parasite and/or pathogen in insects, and the animal pathogen *Coxiella burnetii*. The *Burkholderia* and *Aeromonas* symbionts possibly act in digesting leaf litter as suggested by their close relatives, phenol oxidase- and xylanase-producing bacteria, respectively. However, since none of the closest relatives of symbionts in *A. aquaticus* is known to produce cellulases, we propose that the symbionts described herein do not contribute to the digestion of cellulose by their isopod hosts.

Keywords: Isopoda; Asellota; Hepatopancreatic symbionts; 16S rDNA; *Rhodobacter*; *Burkholderia*; *Aeromonas*; *Rickettsiella*;

5.2 Introduction

The freshwater isopod, *Asellus aquaticus* (Isopoda: Asellota), is one of the most common detritivorous crustaceans in temperate lentic freshwaters. It mainly feeds on decaying plant leaf litter alike its terrestrial relatives, e.g. the common woodlice *Porcellio scaber* and *Oniscus asellus*, *Armadillidium vulgare*, and *Trachelipus rathkii* (Isopoda: Oniscidea). In these terrestrial isopods and in their semi-terrestrial (intertidal) relatives of the genus *Ligia*, hepatopancreatic symbionts have been documented, but not in marine isopod species investigated so far (Wang *et al.*, 2004a; Wang *et al.*, 2004b; Zimmer *et al.*, 2002). It has been proposed that these symbionts serve in producing cellulases and/or phenol oxidases to facilitate the hydrolytic digestion of cellulose and oxidative degradation of phenolic leaf litter compounds by their isopod hosts, which may have been a prerequisite for the colonisation of land and an adaptation to terrestrial food sources by ancestors of recent terrestrial isopods (Zimmer, 2002).

The hepatopancreas (digestive midgut glands) of *A. aquaticus*, consisting of two pairs of tubular caeca, act in the secretion of digestive enzymes and the absorption of nutrients. Zimmer & Bartholmé (2003), through antibiotic treatment and analysis of cellulase and phenol oxidases activity, provided evidence for symbiotic bacteria contributing to digestive processes of *A. aquaticus* by producing cellulases and phenol oxidases (Zimmer and Bartholmé, 2003). However, a proof for their assumption has not been presented, and symbiotic bacteria of *A. aquaticus* could not yet be isolated and cultured *in vitro* (Y. Wang and M. Zimmer, unpubl.). Therefore, little is known about the hepatopancreatic bacteria in this freshwater isopod.

In this study, PCR-based amplification of small subunit ribosomal DNA (16S rDNA), cloning, sequencing, and phylogenetic analysis of the symbionts were performed. Our molecular data not only serve as the first step towards identification of the symbionts but also reveal insight into possible contributions of bacterial symbionts to the digestive physiology of their isopod hosts as well as into the evolution of the symbiosis.

5.3 Materials and Methods

5.3.1 Isopods

Asellus aquaticus was collected in freshwaters in the vicinity of Kiel and in Lake Neuwühren near Plön, Germany, and maintained in aerated tap water at 12°C and fed with mixed leaf litter taken from the field before they were used in the experiment.

5.3.2 DNA extraction

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

5.3.3 PCR

Primers used in this study are shown in Table 1. PCR amplification of Bacterial 16S rRNA genes by using the eubacteria primer pairs S-D-Bact-0063-a-S-21/S-D-Bact-1387-a-A-18 (for samples collected from Plön) and S-D-Bact-0007-a-S-21/S-D-Bact-1492-a-A-22 (for samples collected from Kiel) was carried out as follows: 1 μl of sample DNA, 5 μl of 10 \times PCR buffer, 50 μM of dNTP mixture, 0.5 μM of appropriate primer, and 1 U of thermostable *Taq(E)*⁻ polymerase (Peqlab, Germany) were added in a 0.2 ml PCR tube (Biozym, Germany). The total volume was adjusted to 50 μl with PCR water (Sigma). Thirty cycles of amplification were performed with a Thermal Cycler (Eppendorf), starting the program with a denaturing step of 3 min at 94°C followed by 29 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, and a final extension step at 72°C for 7 min.

Table 1 Primers used in this study

Primer ^a	Primer sequence from 5' to 3'	Reference or source
S-D-Bact-0007-a-S-21	AGAGTTTGATCCTGGCTCAG	(Weisburg <i>et al.</i> , 1991)
S-D-Bact-1492-a-A-22	TACGG(C/T)TACCTTGTTACGACTT	(Weisburg <i>et al.</i> , 1991)
S-D-Bact-0063-a-S-21	CAGGCCTAACACATGCAAGTC	(Marchesi <i>et al.</i> , 1998)
S-D-Bact-1387-a-A-18	GGGCGGWGTGTACAAGGC	(Marchesi <i>et al.</i> , 1998)
S-D-Bact-0515-a-S-19	GTGCCAGCAGCCGCGGTAA	(Henckel <i>et al.</i> , 1999)
S-D-Bact-0907-a-A-15	AATTCCTTTGAGTTT	(Henckel <i>et al.</i> , 1999)
M13F	CAGGAAACAGCTATGAC	Promega
M13R	CGACGTTGTTAAACGACGGCCAGT	Promega

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

Vector PCR for detecting the correct insert of 16S rRNA genes was carried out by using M13 forward and reverse primers with the protocol described above except for using 1 U of thermostable *Taq* polymerase (MBI, Germany) and the appropriate buffer. 27 cycles of amplification were performed as follows: 94°C for 3 min, 55°C for 30 s, 72°C for 1 min (one cycle); 94°C for 30 s, 55°C for 30 s, 72°C for 1 min (26 cycles); followed by a final incubation at 72°C for 4 min.

5.3.4 Cloning and RFLP analysis

PCR products of 16S rRNA genes were purified with the E.Z.N.A Cycle-Pure Kit (Pierce) and subsequently ligated into the pGEM-T vector cloning system (Promega) as described in the manufacturer's instructions. The ligation product was then transformed into competent cells (*Escherichia coli* JM 109) provided in the pGEM-T vector cloning kit. Positive ampicillin-resistant transformants were selected through blue-white screening. The correct sizes of the inserts were confirmed with vector PCR. Amplicons of the correct size were subjected to digestion by using the restriction endonucleases *MspI* and *RsaI* as follows: 18 µl of PCR products, 2 µl of Y⁺/Tango 10× buffer (MBI), and 1 unit of the respective enzyme were added into a sterile tube and incubated at 37°C for 15 h. Aliquots of 10 µl of each digestion were subjected to electrophoresis by using 4% (wt/vol) NuSieve 3:1 agarose (BioWhittaker Molecular applications) gel.

Restriction fragment length polymorphism (RFLP) patterns were compared by eye. The representative clones from each RFLP group were cultured in LB medium containing 100 µg/ml ampicillin, being shaken at 1,400 rpm at 37°C for 10 h. Plasmids were extracted and purified according to the Easy Nucleic Acid Plasmid Miniprep Kit 1 instructions (Peqlab) and subjected to DNA sequencing.

5.3.5 Sequencing of cloned 16S rRNA genes

Double-stranded sequencing of the inserts using primers S-D-Bact-0007-a-S-21 and S-D-Bact-1492-a-A-22, S-D-Bact-0063-a-S-21 and S-D-Bact-1387-a-A-18, as well as S-D-Bact-0515-a-S-19 and S-D-Bact-0907-a-A-15 was performed on an ABI sequencer by GATC (<http://www.gatc.de>).

5.3.6 Sequence assembling and phylogenetic analysis

Sequence assembling was performed using Seqman (DNASTar) (<http://www.dnastar.com>). Comparison with public database was done using BLAST homology search algorithms (Altschul *et al.*, 1997). Phylogeny was analysed using the programs available online at the Ribosomal Database Project (RDP) site (<http://www.cme.msu.edu/RDP/html/analyses.html>) (Cole *et al.*, 2003). Closely related sequences were retrieved from GenBank and added to the alignment. Only sequences with more than 1400 nucleotides were used for the alignment. The 16S rRNA gene sequences from the hepatopancreatic symbionts in *A. aquaticus* were submitted to GenBank under accession numbers AY573580 (clone 12), AY573581 (clone 3-5'), AY573582 (clone 3-3'), AY447040 (clone 7), AY447041 (clone 10) and AY447042 (clone 11).

5.4 Results

5.4.1 Cloning and sequencing

Results of PCR amplification, cloning and RFLP analysis of 16S rRNA genes are summarised in Table 2. PCR amplicons using the primer pair S-D-Bact-0007-a-S-21/S-D-Bact-1492-a-A-22 were obtained only from the DNA samples extracted from hepatopancreas of isopods collected in Kiel. Therefore, the primer pair S-D-Bact-0063-a-S-21/S-D-Bact-1387-a-A-18 was tested for that of isopods collected in Plön, which

resulted in a successful amplification for the 16S rRNA gene. Sequencing of representative clones from each RFLP group (Table 2) yielded 1283 bp for clone 12 and 1255 bp for clone 3, and nearly complete 16S rRNA genes for clone 7 (1494 bp), clone 10 (1479 bp) and clone 11 (1461 bp). Clone 7 and clone 10 had almost identical sequences (99.7% similarity) indicating that they represent two close bacterial lineages.

Table 2 PCR amplification, cloning and RFLP analysis of the 16S rRNA genes from hepatopancreatic bacteria in the freshwater isopod *Asellus aquaticus*

Location	PCR products (kb)	Positive clones ^a	RFLP groups		
			I	II	III
Plön	~1.3	21(15) ^b	8 (12) ^c	7 (3)	–
Kiel	~1.5	43 (37)	15 (7)	13 (10)	9 (11)

^a Ampicillin-resistant and white clones.

^b Number without parenthesis: total number of screened positive transformants; number with parenthesis: number of clones with the correct insert of PCR products.

^c Number without parenthesis: number of clones with identical RFLP profiles; number with parenthesis: selected representative clones for sequencing.

5.4.2 Phylogenetic analysis

Comparison with public databases of 16S rRNA gene sequences revealed that clones 3 and 12 were closely related to members of the bacterial genera *Aeromonas* (γ -Proteobacteria, similarity >99%) and *Rhodobacter* (α -Proteobacteria, similarity >95%), respectively; Clones 7 and 10 yielded >96% similarities with sequences from the γ -Proteobacterial genus *Rickettiella*; Clone 11 exhibited high similarities (>99%) to sequences from *Burkholderia*, β -subclass of Proteobacteria (Table 3). Phylogenetic analysis corroborated the results of homology comparison. Clone 12 was grouped within the genus *Rhodobacter* of α 3-Proteobacteria, represented a sister lineage of *Rhodobacter apigmentum*, *R. capsulatus* and *R. sphaeroides*, and was distantly related to other groups in α -Proteobacteria (Fig. 1A). Clone 3 belonged to the genus *Aeromonas* and clustered with *Aeromonas sobria* within the same evolutionary branching order (Fig. 1B). Clones 7 and 10 fell within the *Coxiella burnetii* subgroup of the *Legionella* group and clustered closely with *Rickettsiella grylli*, but distantly with

the genera *Coxiella* and *Legionella* (Fig. 1B). Clone 11 was placed within the *Burkholderia glathei* subgroup of the *Burkholderia* group and grouped with free-living *Burkholderia* species, but was distantly related to parasitic and pathogenic members in *B. cepacia* and *B. thailandensis* subgroups (Fig. 1C). Both neighbour-joining and weighbor-joining (Bruno *et al.*, 2000) analysis revealed the same phylogenetic affiliation of the clones obtained from the hepatopancreas of *A. aquaticus* and the same tree topology and branch order as shown in Figure 1.

Table 3 Sequence analysis of 16S rRNA genes of clones obtained from the hepatopancreatic bacteria of *Asellus aquaticus*

Location	Clone no.	Sequence affiliation		Most closely related bacterial sequence	% Identity
		Subclass	Genus		
Plön	12	α -Proteobacteria	<i>Rhodobacter</i>	<i>Rhodobacter apiqmentum</i>	95.0
	3-5'	γ -Proteobacteria	<i>Aeromonas</i>	<i>Aeromonas sobria</i>	99.7
	3-3'	γ -Proteobacteria	<i>Aeromonas</i>	<i>Aeromonas sobria</i>	99.8
Kiel	7	γ -Proteobacteria	<i>Rickettsiella</i>	<i>Rickettsiella grylli</i>	96.5
	10	γ -Proteobacteria	<i>Rickettsiella</i>	<i>Rickettsiella grylli</i>	96.7
	11	β -Proteobacteria	<i>Burkholderia</i>	<i>Burkholderia</i> sp. N2P5	99.9

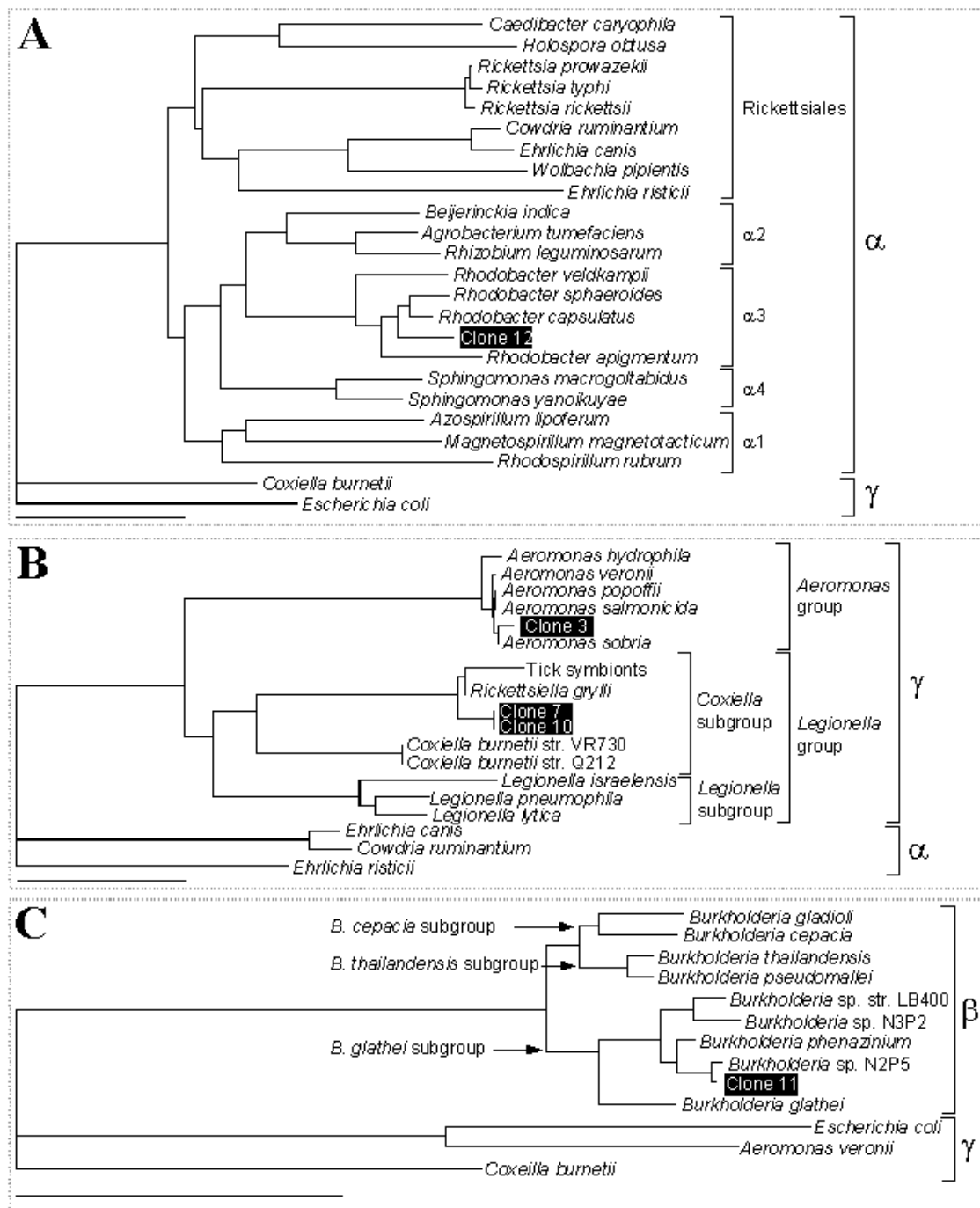


Fig. 1. Rooted phylogenetic tree (neighbour-joining) of the 16S rDNA sequences retrieved from hepatopancreatic symbionts of the freshwater isopod, *Asellus aquaticus*, and their closest relatives and reference strains as inferred from 16S rDNA sequence data. Distances were calculated using the Kimura 2-parameter algorithm. The tree is rooted with *Coxiella burnetii* and *Escherichia coli* in (A), *Ehrlichia canis*, *Cowdria ruminantium* and *Ehrlichia risticii* in (B), and *Escherichia coli*, *Aeromonas veronii* and

Coxeilla bumetii in (C). Clones (3, 12, 7, 10, and 11) obtained from *A. aquaticus* are shown in white bold on black ground. The branches of the α -, β -, and γ -subclasses of Proteobacteria as well as related species groups and subgroups are marked in the tree. The scale bar represents 0.05 substitutions per nucleotide position.

5.5 Discussion

The present phylogenetic analysis of 16S rRNA genes demonstrates that hepatopancreatic symbionts in the freshwater isopod, *A. aquaticus*, occurred in high diversity between populations collected in Plön and Kiel, Germany, which possibly results from the geographic distribution of the isopod hosts.

5.5.1 *Rhodobacter* sp. symbionts

The *Rhodobacter* group belongs to α -3 Proteobacteria, and its members exhibit extraordinary metabolic versatility and flexibility (Imhoff, 2001). So far, only one symbiotic bacterium of the genus *Rhodobacter* was found that lives in intimate association with an invertebrate host, the gutless deep-sea tubeworm *Lamellibrachia* sp., and appears to contribute to the primary production of its host (Elsaied *et al.*, 2002). However, it represents a lineage only distantly related (<89% similarity) to the *Rhodobacter* symbionts we found in *A. aquaticus*. The closest relatives of *A. aquaticus* symbionts are free-living freshwater *Rhodobacter* spp. Thus, the present symbionts have probably been acquired from *A. aquaticus* habitats and represent a unique symbiotic lineage in the genus *Rhodobacter*.

5.5.2 *Aeromonas* sp. symbionts

Species of the bacterial genus *Aeromonas* are ubiquitous in freshwater environments (Janda and Abbott, 1998; Sugita *et al.*, 1995). The species most closely related to the *A. aquaticus* symbionts we found, *Aeromonas sobria* (>99.7% similarity), has been recognised as opportunistic pathogen in fish and human (Janda and Abbott, 1998; McGarey *et al.*, 1991), suggesting that the present symbiotic bacteria derived from pathogens, and isopod hosts were infected after the colonisation of freshwater habitats. This conclusion corroborates the acquisition of bacterial symbionts in the course of the

evolutionary shift to feeding on terrestrial food sources as proposed by (Zimmer and Bartholmé, 2003). In addition, *Aeromonas caviae* ME-1 (Suzuki *et al.*, 2001) and *Aeromonas caviae* W-61 (Roy *et al.*, 2000), sharing >97% similarity with *A. asellus* symbionts, are able to produce xylanases. Accordingly, *Aeromonas* symbionts might aid *A. aquaticus* in the digestion of leaf litter (cf. Zimmer and Bartholmé, 2003).

5.5.3 *Burkholderia* sp. symbionts

The *Burkholderia* group contains important pathogens and parasites of humans, animals and plants, e.g., species of the *B. cepacia* and *B. thailandensis* subgroups (Fig. 1C), as well as organisms useful in promoting plant growth and bioremediation, e.g., species in the *B. glathei* subgroup (Fig. 1C) (Woods and Sokol, 2001). Close relatives of the β -Proteobacterial symbionts of *A. aquaticus*, *Burkholderia* sp. N2P5 and *B.* sp. N3P2 (>98% similarity), are able to degrade polycyclic aromatic hydrocarbons by an oxygenase (Mueller *et al.*, 1997). Additionally, carbohydrate-, amino acid- and organic acid-oxidase activity were detected in *B. phenazinium* (>98% similarity) (Viallard *et al.*, 1998). Given that a phylogenetic relationship between close strains mirrors phenotypic similarity (Moore *et al.*, 1998), the *Burkholderia* symbionts of *A. aquaticus* are possibly able to oxidise phenolic compounds of the leaf litter. This implication appears to support the finding that hepatopancreatic symbionts serve in producing phenol oxidases to contribute to the digestion of leaf litter by their isopod host (Zimmer and Bartholmé, 2003).

5.5.4 *Rickettsiella* sp. symbionts

Rickettsiella grylli was first described as an intracellular parasite of *Gryllus bimaculatus* and related species of crickets by Vago and Martoja (Vago and Martoja, 1963), and was affiliated with other two *Rickettsiella* species, *R. popilliae* and *R. chironomii*, to the order *Rickettsiales* (α -Proteobacteria) solely on the basis of the close association of these organisms with arthropods and their intra-vacuolar colonization (Weiss and Moulder, 1984). Roux *et al.* (1997) reclassified it to the γ -Proteobacteria, most closely related to *Coxiella burnetii* and *Legionella* sp. by comparing analysis of the 16S rRNA gene sequence (Roux *et al.*, 1997). To date, although Kurtti *et al.* (2002) documented an intra-cellular *Rickettsiella*-like symbiotic bacterium infecting the female tick, *Ixodes*

woodi (Ixodidae) based on cloning and sequence analysis of the 16S rRNA gene (Kurtti *et al.*, 2002), the novel *Rickettsiella*-like symbionts found in a crustacean host herein represent the closest relatives of *R. grylli* (Fig. 1B), which broadens the host spectrum of the genus *Rickettsiella*.

Zimmer & Bartholmé (2003) proposed that hepatopancreatic bacteria in *A. aquaticus* are involved in the hydrolysis of cellulose in leaf litter. However, phylogenetic analyses of these hepatopancreatic symbionts we found in the presented study only suggest xylanases and phenolic oxidases. Cellulose-degrading capability has not been documented in any of the close relatives of these hepatopancreatic symbionts (Lynd *et al.*, 2002). Based on DAPI-staining and methods of molecular biology as used in this study, we detected neither bacteria nor bacterial DNA in a detritivorous amphipod, *Gammarus pulex* (Crustacea: Amphipoda) (Y. Wang, A. Brune & M. Zimmer, unpubl.), living in the same freshwater habitats as *A. aquaticus* and also feeding on leaf litter (Zimmer and Bartholmé, 2003). Thus, feeding on terrestrial leaf litter by crustaceans does not necessarily require bacterial symbionts. We expect the symbionts of *A. aquaticus* described herein to be deficient of cellulase. However, we cannot exclude the possibility of other bacterial symbionts that do provide cellulases to their host. Overall, the high diversity of symbionts in *A. aquaticus* hint on different interactions of these symbionts with their isopod host with respect to digestive and nutritive physiology.

In conclusion, our study clearly demonstrates that the freshwater isopod, *A. aquaticus*, harbours diverse hepatopancreatic symbionts. The hepatopancreatic bacteria were possibly acquired several times independently and exchanged or lost after their isopod hosts colonized and adapted to freshwater habitats. Phylogenetic analysis of the hepatopancreatic symbionts hint on the potential role of at least some of these symbionts in facilitating digestion by their isopod hosts. However, whether this association between hepatopancreatic bacteria and their isopod hosts involves the digestion of cellulose in leaf litter and hence plays a role in host adaptation to evolutionarily new food sources remains to be explored.

5.6 References

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6 Geographic distribution of hepatopancreatic bacterial symbionts in natural populations of terrestrial isopods (Isopoda: Oniscidea)

6.1 Abstract

Bacterial symbionts, the candidate species *Hepaticola porcellionum* and *Hepatoplasma crinochetorum* have been identified in the hepatopancreas of terrestrial isopods *Porcellio scaber* and *Oniscus asellus*. An extensive and systematic survey for the geographic distribution and infection frequency of these two symbiotic bacteria in different terrestrial isopod species was performed using fluorescence-labelled oligonucleotide probe hybridisation and diagnostic PCR techniques. They represent the major symbiotic bacteria in the terrestrial isopods examined. Double infections with both of them in a single isopod individual were not observed. Both of them were found in all of 6 *P. scaber* populations (n = 177) collected from geographically distant localities, Germany (Kiel and Köln) and Canada (Bamfield, Vancouver Islands and Haines Islands), in 3 *O. asellus* populations (n = 60) sampled in geographically distant sites in Germany (Kiel and Köln), and in *Trachelipus rathkii* from Kiel. In addition, *Candidatus* *Hepatoplasma crinochetorum* was also detected in French *O. asellus*, in German *Philoscia muscorum* and *Armadillidium vulgare*, and in Canadian *Alloniscus perconvesus*. Although their infection frequency varied within and between isopod populations, the symbiont *Candidatus* *Hepatoplasma crinochetorum* was overall more prevalent than *Candidatus* *Hepaticola porcellionum*. Aposymbiotic isopod individuals were found in all of these six isopod species investigated with different frequency between populations. Incidence of isopod individuals with high cell density ($>10^7$ cells per individual) of symbionts was low (<40%) in most populations of *P. scaber* and *O. asellus*. The distribution and infection rates of the symbionts appear not to be affected

by the season. However, in comparison with winter and spring, the frequency of individuals harbouring high cell numbers of the symbionts is much higher in summer and autumn.

6.2 Introduction

Terrestrial isopods (Isopoda: Oniscidea) represent the only taxon within the Crustacean that consequently colonized land, rendering them the ideal model organisms to explore evolutionary mechanisms involving morphological and eco-physiological adaptations to the terrestrial habitats. One of the most intriguing features of terrestrial isopods is the dense population of microorganisms inhabiting the hepatopancreas (midgut glands), which might have facilitated isopod host to adapt to and digest terrestrial food (e.g., leaf litter) (Zimmer, 2002). However, most previous work on hepatopancreatic symbionts was restricted to morphological description of hepatopancreatic microorganisms. Recently, we reported that two types of phylogenetically distinct hepatopancreatic symbionts, the candidate species 'Hepatincola porcellionum', curved rod-shaped *Rickettia*-like bacteria (α -subdivision of Proteobacteria), and 'Hepatoplasma crinochetorum', small and large sphere-shaped *Mycoplasma*-like bacteria (Mollicutes), colonize the wall of the hepatopancreatic ceca of different individuals of the isopod *Porcellio scaber* (Wang *et al.*, 2004a; Wang *et al.*, 2004b). Interestingly, close relatives of these bacteria were also found in the terrestrial isopod *Oniscus asellus* (Wang *et al.*, 2004c), suggesting that these two species of symbionts occur in different terrestrial isopod species. However, the discrepancies among previous morphological descriptions of bacteria in the midgut glands of several isopods (Donadey and Besse, 1972; Hames and Hopkin, 1989; Wood and Griffith, 1988) suggest that different isopod species, and possibly even different populations of the same species, are colonized by different symbionts. Thus, in the present study, an extensive survey of these two bacterial symbionts among geographically distant natural isopod populations was performed. Our data reveal the intra- and inter-specific distribution profile of these symbionts, and provide valuable insights into the evolution of the symbiosis.

6.3 Materials and methods

6.3.1 Isopod collections

Isopod samples used for surveying the geographical distribution of hepatopancreatic symbionts are listed in Table 1. Each collection site was considered a different isopod population, as they were separated from one another by >1 km. At each locality, about 10 m² area was set to collect isopod living beneath decaying wood, or in soils (marsh grass habitat), or in sands or gravel (beach habitat). Hepatopancreas of the specimens were dissected immediately and prepared for DAPI (4',6-diamidino-2-phenylindole) staining and fluorescence *in situ* hybridization (FISH) as described in Wang et al. (Wang *et al.*, 2004b). Both female and male adults were used in the study.

Table 1 Samples of different species and populations of terrestrial isopods collected from Germany, Canada, and France

Sample	Locality	Population ID	Date
<i>Porcellio scaber</i>	Wood, Kiel, Germany	Ps-1 (n=22)	May and Jun. 2002
	Marsh grass, Kiel, Germany	Ps-2 (n=70)	May and Jun. 2002
	Wood, Köln, Germany	Ps-3 (n=9)	Jun. 2002
		Ps-4 (n=15)	Jun. 2002
	Bamfield, Vancouver Island, Canada	Ps-5 (n=24)	Aug. 2003
	Haines Island, Canada	Ps-6 (n=37)	Aug. 2003
<i>Oniscus asellus</i>	Wood, Kiel, Germany	Oa-1 (n=35)	May and Jun. 2002
	Wood, Köln, Germany	Oa-2 (n=14)	Jun. 2002
		Oa-3 (n=11)	Aug. 2002
	Forêt de qizet, France	Oa-4 (n=10)	May 2003
<i>Philoscia muscorum</i>	Wood, Kiel, Germany	Pm-1 (n=13)	Jun. 2003; Mar. 2004
<i>Armadilidium vulgare</i>	Wood, Kiel, Germany	Av-1 (n=11)	May, 2003
<i>Trachelipus rathkii</i>	Wood, Kiel, Germany	Tr-1 (n=8)	Feb. and May, 2003
<i>Alloniscus perconvexus</i>	Pachena Beach, Vancouver Island, Canada	Ap-1 (n=16)	Aug. 2003

In order to estimate whether or not environmental factors affect the prevalence of the hepatopancreatic symbionts, 12 subsamples of a population of *Porcellio scaber* collected separately from decaying wood in every months (Jan. to Dec., 2003) were investigated (Table 2).

Table 2 *Porcellio scaber* sampled for the analysis of seasonal prevalence of hepatopancreatic symbionts

Individual number	8	9	9	28	10	10	12	11	12	12	12	19
Sampling date (2003)	Jan. 22	Feb. 25	Mar. 26	Apr. 24	May 24	Jun. 20	Jul. 21	Aug. 21	Sep. 24	Oct. 27	Nov. 19	Dec. 20

6.3.2 FISH

Different specific fluorescently labelled probes used in this study are listed in Table 3. Hybridisation using probes EUB338, ALF1b and PsSym120 were performed to detect the *Rickettsia*-like symbiont, *Candidatus* Hepatocola porcellionum according to Wang et al. (Wang *et al.*, 2004b); double hybridisation using probes EUB338 and PsSym352 were used to detect the *Mycoplasma*-like symbiont, *Candidatus* Hepatoplasma crinochetorum as described by Wang et al. (Wang *et al.*, 2004a).

Table 3 Fluorescence-labelled oligonucleotide probes used in this study

Probes ^a	Probe sequence from 5' to 3'	Target site ^c (rRNA position)	[%] FA ^d in situ	Reference
NONEUB	ACTCCTACGGGAGGCAGC		35	(Wallner <i>et al.</i> , 1993)
EUB338	GCTGCCTCCCGTAGGAGT	16S (338-355)	0-35	(Amann <i>et al.</i> , 1990)
ALF1B	CGTTCGYTCTGAGCCAG	16S (19-35)	20	(Manz <i>et al.</i> , 1992)
GAM42a	GCCTTCCCACATCGTTT GCCTTCCCCTTCGTTT ^b	23S (1027-1043)	35	(Manz <i>et al.</i> , 1992)
PoSsym352	GTGAAAAATTCCCTACTGCTG	16S (352-373)	0-20	(Wang <i>et al.</i> , 2004a)
PoSsym120	AGCCAAATCCCACGTGT	16S (120-138)	20	(Wang <i>et al.</i> , 2004b)

^a 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](http://www.interactiva.de)). NONEUB, negative control probe complementary to EUB338; EUB338, most Eubacteria; ALF1B, α -Proteobacteria, some δ -Proteobacteria, Spirochaetes; GAM42a, γ -Proteobacteria; PsSym352, the candidate species, *Hepatoplasma crinochetirum*; PsSym120, the candidate species, *Hepatincola porcellionum*.

^b Competitor probe BET42a.

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

^d Percent amount of formamide (vol/vol) in the hybridisation buffer.

6.3.3 DNA extraction and diagnostic PCR

Hepatopancreas of randomly chosen isopod individuals from each population listed in Table 1 were subjected to DNA extraction following the protocol provided by DNeasy[®] Tissue kit (Qiagen). The purified DNA was eluted in 100 μ l of AE buffer provided in the kit.

PsHp138f (5'-ACA CGT GGG AAT TTG GCT-3'; *Escherichia coli* positions 120-138, complementary sequences of the specific probe PsSym120 (Wang *et al.*, 2004b), was used as a *Candidatus* *Hepatincola porcellionum*-specific PCR forward primer with the bacteria-specific reverse primer 1492r (Weisburg *et al.*, 1991); PsHc372f (5'-CAG CAG TAG GGA ATT TTT CAC-3'; *Escherichia coli* positions 352-372, complementary sequences of the specific probe PsSym352 (Wang *et al.*, 2004a), was used as a *Candidatus* *Hepatoplasma crinochetorum*-specific PCR forward primer with the bacteria-specific reverse primer 1492r (Weisburg *et al.*, 1991). The specificity of the primer sets PsHp138f-1492r and PsHc372f-1492r was determined in a series of amplification reactions with a variety of nonhomologous templates extracted from one bacteria strain and from symbiont-harbouring hepatopancreas of other members of the semi-terrestrial isopod species, *Ligia oceanica*, *Ligia pallasii*, *Tylos ponticus* and the freshwater isopod species, *Asellus aquaticus*.

PCR amplification was performed in a 200 μ l PCR tube filled with 50 μ l reaction volume containing 200 μ M of each dNTP, 5.0 μ l of a 10 \times reaction buffer (Eppendorf), 25 pmol of each primer, 1.0 U of Taq DNA polymerase (Eppendorf), and 1 μ l 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. Aliquots of 5 µl of the PCR products were resolved in 1.0% (wt/vol) agarose gels in 1.0× TAE buffer (40 mM Tris-acetate, 1mM EDTA). The gels were visualized under ultraviolet light after ethidium bromide-staining (0.5 µg/ml).

DNA extracted from symbiont-free hepatopancreas of isopod species and from symbiont-harboring hepatopancreas of *Porcellio scaber* (Wang *et al.*, 2004a; Wang *et al.*, 2004b) as well as *Oniscus asellus* (Wang *et al.*, 2004c) for cloning and sequencing were used as negative and positive controls for the diagnostic PCR amplifications, respectively.

6.4 Results

6.4.1 General description of the symbionts *Candidatus Hepatincola porcellionum* and *Candidatus Hepatoplasma crinochetorum* in different isopod populations

All curved rod-shaped hepatopancreatic symbiotic bacteria detected in different individuals within different populations of different isopod species collected from different localities hybridised with probe EUB338 (figures not shown), probe ALF1b and the specific probe PsSym120 (Fig. 1A), suggesting that they represent close relatives of the symbiont *Candidatus Hepatincola porcellionum*. All sphere-shaped (both small and large cell types) hepatopancreatic symbiotic bacteria found in different individuals within different populations of different isopod species collected from different localities hybridised with probe EUB338 and the specific probe PsSym352 (Fig. 1B), suggesting that they are close relatives of the symbiont *Candidatus Hepatoplasma crinochetorum*. Further analysis with diagnostic PCR revealed identical results (Fig. 2).

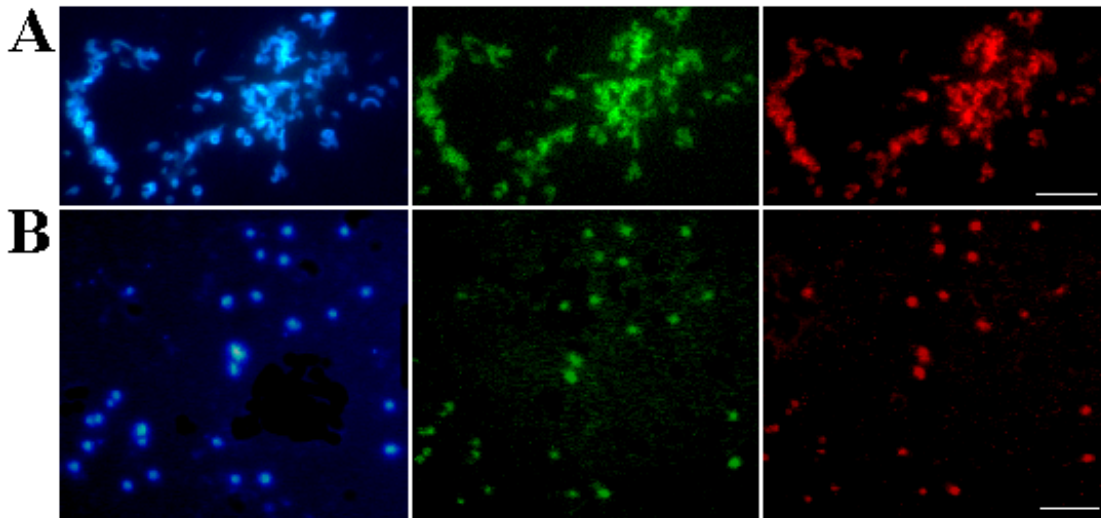


Figure 1 (A) One representative of the curved rod-shaped symbiotic bacteria in the midgut gland homogenate of different isopod species examined in this study. The three epifluorescence photomicrographs show the same microscopic field of a preparation stained with DAPI (blue colour) and double-hybridized with oligonucleotide probes specific for α -Proteobacteria (ALF1b, green colour) and *Candidatus Hepatincola porcellionum* (PsSym120, red colour). Scale bar, 5 μm . (B) one representative of the sphere-shaped symbiotic bacteria in the midgut gland homogenate of different isopod species examined in this study. The three epifluorescence photomicrographs show the same microscopic field of a preparation stained with DAPI (blue colour), and double-hybridized with oligonucleotide probes specific for Bacteria (EUB338, green colour) and *Candidatus Hepatoplasma crinochetorum* (PsSym352, red colour). Scale bar, 5 μm .

6.4.2 Hepatopancreatic symbiont composition within a single individual

Either the candidate species *Hepatincola porcellionum* or *Hepatoplasma crinochetorum* harboured in a single isopod individual represented homogeneous population based on FISH hybridisation (Fig. 1). Co-infected isopod individuals with both types of symbionts were not found. The composition of cell types of *Candidatus Hepatoplasma crinochetorum* within a single isopod individual exhibited high diversity. Large spherical cells, small spherical cells, and mixed large and small spherical cells colonized different individuals. Their infection rates were markedly different within and between isopod populations collected from diverse localities (Fig. 3).

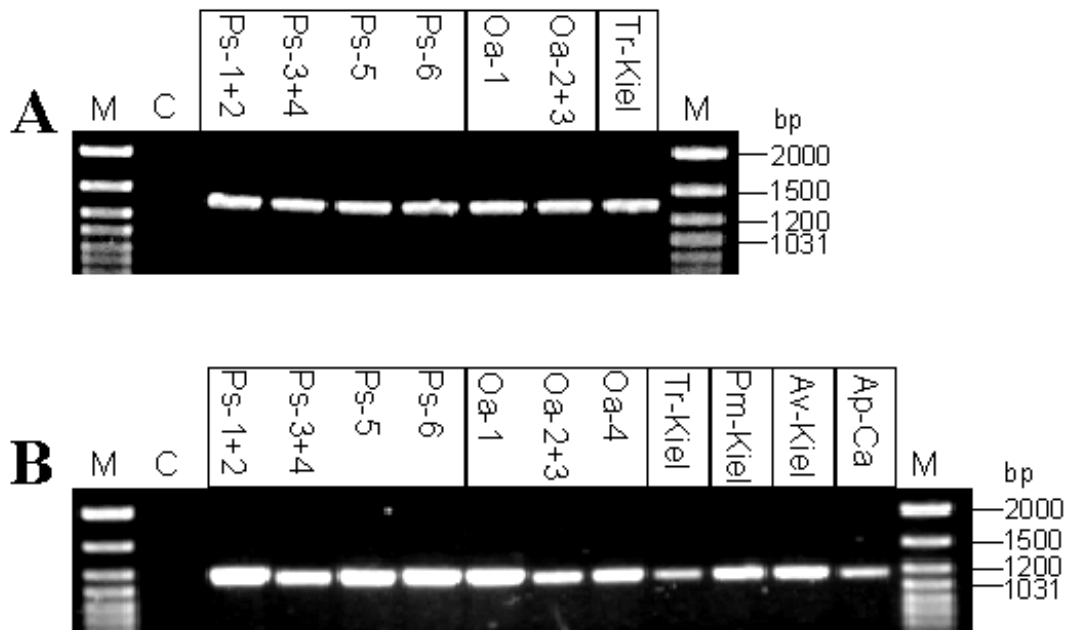


Figure 2 Diagnostic PCR amplicons (A) ca. 1,412 bp of 16S rRNA genes from curved rod-shaped symbiotic bacteria using primers 138F/1492R specific for *Candidatus Hepatincola porcellionum*; (B) ca.1,162 bp of 16S rRNA genes from sphere-shaped symbiotic bacteria using primers 372F/1492R specific for *Candidatus Hepatoplasma crinochetorum*, found in different isopod populations. M, markers; C, negative control; Population ID refers to Table 1.

6.4.3 Prevalence of symbionts in *P. scaber* and *O. asellus*

In order to assess the distribution of symbionts, *P. scaber* collected from decaying wood (Ps-1) and marsh grass (Ps-2), Kiel, Germany, respectively, were subjected to survey. In both habitats, all detected individuals harboured symbionts, either *Candidatus Hepatoplasma crinochetorum* or *Candidatus Hepatincola porcellionum* (Fig. 3A), indicating that these two symbiotic bacteria are the main symbionts colonizing the hepatopancreas of isopod hosts. Overall, *Candidatus Hepatoplasma crinochetorum* was more prevalent than *Candidatus Hepatincola porcellionum* (59.1% vs. 40.9% in Ps-1; 92.8% vs. 7.2% in Ps-2) (Fig. 3A). In order to evaluate the geographical distribution and frequency of *Candidatus Hepatincola porcellionum* and *Candidatus Hepatoplasma crinochetorum*, two populations from Köln, Germany, Ps-3 and Ps-4, and two populations from Canada, Ps-5 (Vancouver Island) and Ps-6 (Haines Island) were investigated. Both symbionts were detected in these four populations (Fig. 3A); no other

hepatopancreatic bacteria were found. The percentage of isopods hosting one or the other symbiont differed within and between populations (Fig. 3A). However, like in Ps-1 and Ps-2, *Candidatus* Hepatoplasma crinochetorum was the dominant one (Fig. 3A). In contrast to 100% infection in Ps-1, Ps-2, Ps-3 and Ps-4, 75% and 86.5% of individuals in Ps-5 and Ps-6 were aposymbiotic, respectively (Fig. 3A).

In *O. asellus*, population Oa-1 collected in Kiel, Germany, was characterized by high diversity of the distribution frequency of the symbionts. Both symbionts were detected. Consistent with the results on *P. scaber*, *Candidatus* Hepatoplasma crinochetorum was the prevalent symbiont (Fig. 3C). Additionally, 14.3% of individuals harboured short rod-shaped γ -Proteobacteria that hybridised with probes EUB338 and GAM42a (Figures not shown); 31.4% of individuals were aposymbiotic (Fig. 3C). In two populations from Köln, Germany (Oa-2 and Oa-3), both *Candidatus* Hepatincola porcellionum and *Candidatus* Hepatoplasma crinochetorum were observed (Fig. 3C). However, the prevalence of *Candidatus* Hepatincola porcellionum was greater than that of *Candidatus* Hepatoplasma crinochetorum in Oa-2 (42.9% vs. 35.7%) (Fig. 3C). In population Oa-2, 28.6% of individuals were free of any hepatopancreatic bacteria (Fig. 3C). As shown in Fig. 3C, *Candidatus* Hepatoplasma crinochetorum was the unique symbiont in 90% of individuals of population Oa-4 sampled in France; the other 10% of individuals was aposymbiotic.

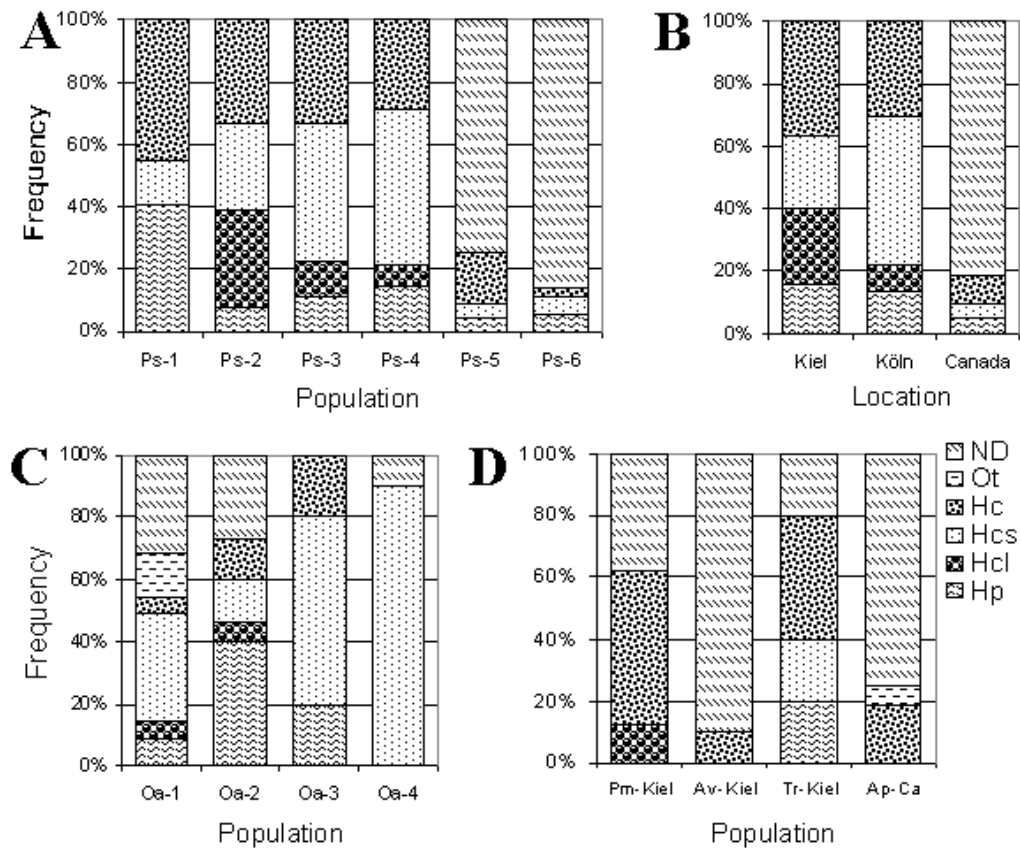


Figure 3 Prevalence of the symbionts *Candidatus* *Hepatincola porcellionum* and *Candidatus* *Hepatoplasma crinocheterum* in 6 populations of *Porcellio scaber* (A and B), in 4 populations of *Oniscus asellus* (C), and in the terrestrial isopod species *Philoscia muscorum* (Pm-Kiel), *Armadilidium vulgare* (Av-Kiel), *Trachelipus rathkii* (Tr-Kiel) (D), and *Alloniscus perconvexus* (Ap-Ca). Population ID refers to Table 1. Hp, *Candidatus* *Hepatincola porcellionum*; Hcl, large sphere of *Candidatus* *Hepatoplasma crinocheterum*; Hcs, small sphere of *Candidatus* *Hepatoplasma crinocheterum*; Hc, both large and small sphere of *Candidatus* *Hepatoplasma crinocheterum*; Ot, other symbiotic bacteria; ND, not detected.

6.4.4 Hepatopancreatic symbionts in different terrestrial isopod species

Small and large sphere-shaped symbiotic bacteria were also observed in *Philoscia muscorum*, *Armadilidium vulgare*, *Trachelipus rathkii*, and *Alloniscus perconvexus*. They hybridised with probe EUB338 and the specific probe PsSym352 (Figures not shown) and were detected by diagnostic PCR amplification (Fig. 2B), suggesting that they are close relatives of *Candidatus* *Hepatoplasma crinocheterum*. The infection rates

of this symbiont were high and similar (>60%) to each other in isopod populations Pm-Kiel and Tr-Kiel, but lower than 20% in Av-Kiel and Ap-Ca (Fig. 3D). Curved rod-shaped symbionts were only found in *T. rathkii*, determined by both FISH (probe EUB338, probe ALFlb and the specific probe PsSym120) (Figures not shown) and diagnostic PCR (Fig. 2A), indicating that they are close relatives of *Candidatus* Hepatincola porcellionum. Aposymbiotic individuals were detected in all these four species with different frequencies (Fig. 3D). Additionally, rod-shaped hepatopancreatic bacteria were observed in 6.3% of individuals of Ap-Ca (Fig. 3D).

6.4.5 Symbiont density

Cell density of *Candidatus* Hepatoplasma crinochetorum and *Candidatus* Hepatincola porcellionum ranged from 10^4 to 10^9 cells per individual in different host individuals. Their distribution differed greatly within and between isopod populations of *P. scaber* (Fig. 4A) and *O. asellus* (Fig. 4B), respectively. The abundance of isopod individuals with high cell density ($>10^7$) of either *Candidatus* Hepatoplasma crinochetorum or *Candidatus* Hepatincola porcellionum were lower than 50% in most populations of *P. scaber* (4 out of 6 populations) (Fig. 4A). In populations Ps-3 and Ps-4, isopod individuals with more than 10^7 of symbiotic bacteria were not found (Fig. 4A). In *O. asellus*, 75% individuals had less than 10^7 of *Candidatus* Hepatoplasma crinochetorum among 3 out of 4 populations (Fig. 4B). In general, taking *Candidatus* Hepatincola porcellionum and *Candidatus* Hepatoplasma crinochetorum as a whole, the abundance of isopod individuals with high density ($>10^7$) of symbionts was low (<40%) in most populations of both *P. scaber* (4 out of 6) (Fig. 4C-a) and *O. asellus* (3 out of 4) (Fig. 4C-b).

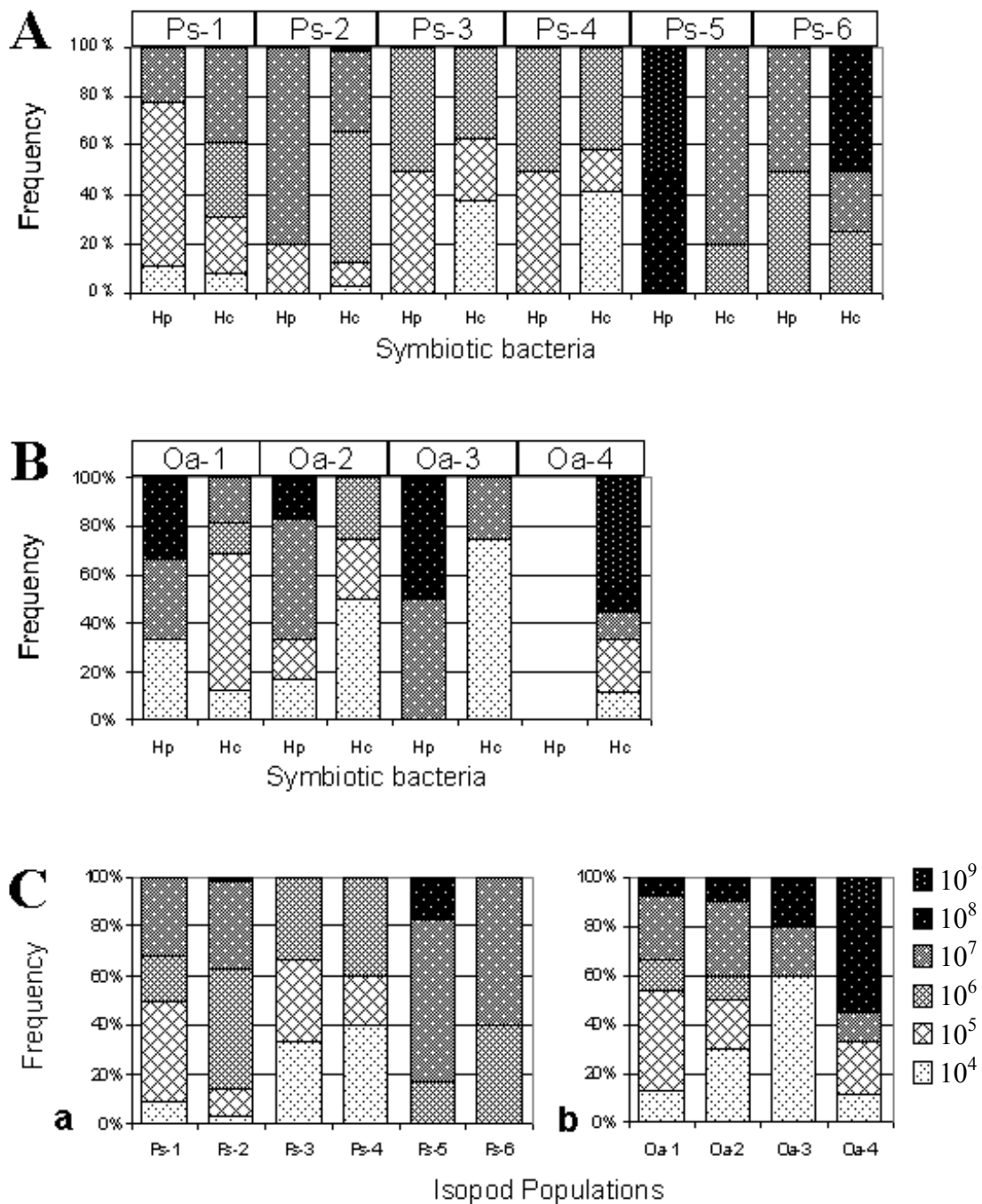


Figure 4 Infection frequency of *Porcellio scaber* (A) and *Oniscus asellus* (B), individuals with different cell numbers of *Candidatus Hepatincola porcellionum* and *Candidatus Hepatoplasma crinochetorum*, respectively. Cell density distribution of both *Candidatus Hepatincola porcellionum* and *Candidatus Hepatoplasma crinochetorum* in different isopod populations *Porcellio scaber* (C-a) and *Oniscus asellus* (C-b). Hp: *Candidatus Hepatincola porcellionum*; Hc: *Candidatus Hepatoplasma crinochetorum*.

6.4.6 Seasonal occurrence of hepatopancreatic symbionts

Candidatus Hepatoplasma crinochetorum was found in all isopod populations collected at every date and represented the most prevalent symbionts (Fig. 5A). *Candidatus* Hepatincola porcellionum occurred in <25% of individuals, but was found in most populations during the entire experimental period (8 out of 12 months) (Fig. 5A). The infection rates by these two symbionts normally ranged from 40% to 60% in 8 out of 12 monthly samplings (Fig. 5A). Additionally, individuals harbouring other symbiotic bacteria, e.g. rod-shaped, long rod-shaped in chain, or helical bacteria, were observed in 50% of all samples (Fig. 5A). Their infection rates varied markedly (range, 3.6% to 25%) (Fig. 5A).

Figure 5B shows the frequency of different cell numbers of *Candidatus* Hepatincola porcellionum and *Candidatus* Hepatoplasma crinochetorum over time. The lowest frequency was found in isopod populations collected in winter. In both populations, the frequency of isopods with high symbiont density ($>10^7$) was as low as 25% in December and January (Fig. 5B). In April and May, the percentage of isopods with more than 10^7 symbionts was some higher but still below 37.5%. By contrast, more than 54% of individuals sampled in summer and autumn harboured high density of symbionts ($>10^7$). Moreover, the highest frequency (>71%) was found in October.

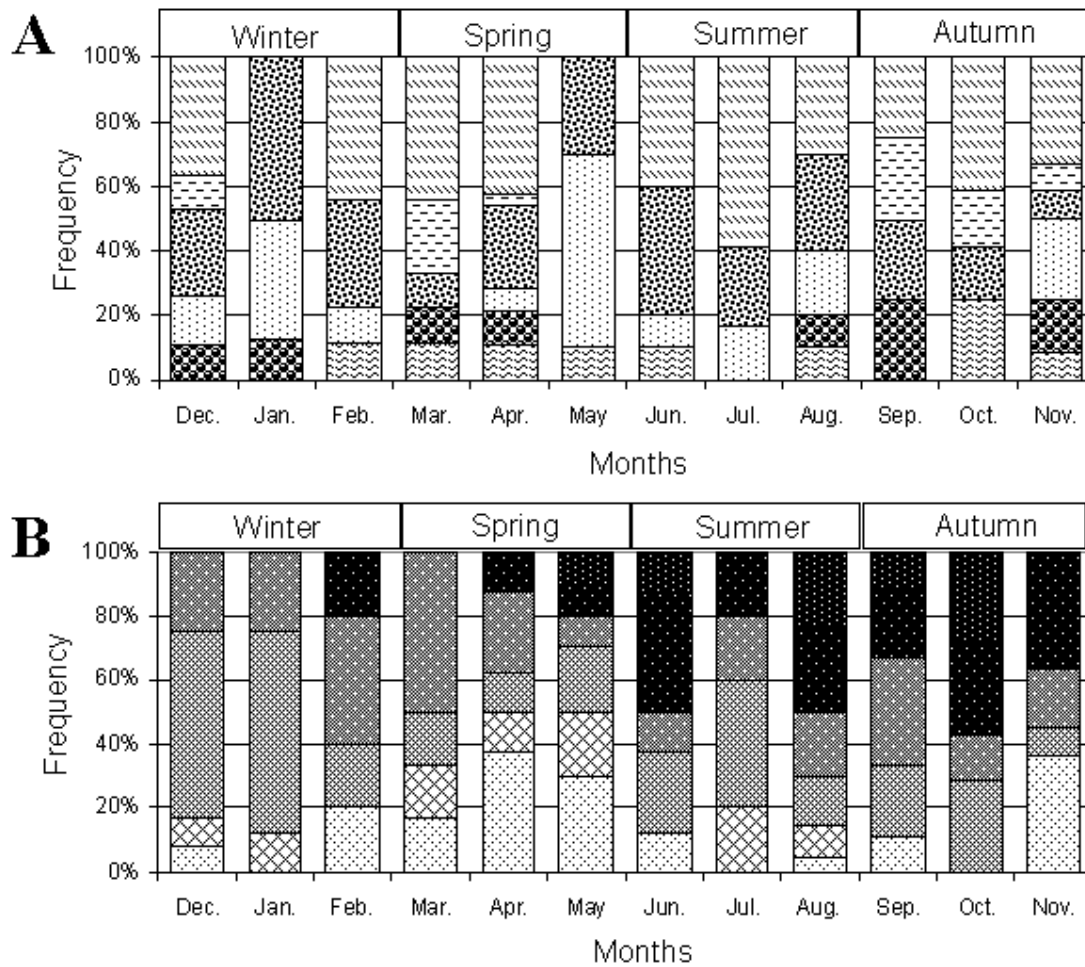


Figure 5 Seasonal infection frequency (A) and cell density distribution (B) of *Candidatus Hepatincola porcellionum* and *Candidatus Hepatoplasma crinochetorum* in *Porcellio scaber*. Symbols as in figures 3 and 4.

6.5 Discussion

Based on FISH and diagnostic-PCR analyses of 16S rRNA genes from bacterial symbionts of isopod hosts, this study provide information on the intra- and inter-population as well as intra- and inter-specific distribution and infection frequency of the hepatopancreatic symbionts, *Candidatus Hepatincola porcellionum* (Wang *et al.*, 2004b) and *Candidatus Hepatoplasma crinochetorum* (Wang *et al.*, 2004a), in natural populations of terrestrial isopod from different geographical locations.

6.5.1 Intra- and inter-population distribution of the symbionts *Candidatus* Hepatoplasma crinochetorum and *Candidatus* Hepatincola porcellionum

In the present study, an ubiquitous distribution of *Candidatus* Hepatoplasma crinochetorum and *Candidatus* Hepatincola porcellionum was demonstrated. *Candidatus* Hepatoplasma crinochetorum has been detected in *P. scaber* and *O. asellus*, not only in Germany, (Kiel and Köln), and in Canada (*P. scaber*) and France (*O. asellus*); they occurred in both *P. scaber* and *O. asellus*, as well as four other terrestrial isopod species populations, *P. muscorum*, *A. vulgare*, *T. rathkii*, and *A. perconvexus* (Fig. 3D). *Candidatus* Hepatincola porcellionum was also common in geographically distant populations of *P. scaber* and *O. asellus* (Fig. 3A-C), and was also found in *T. rathkii* (Fig. 3D). *Candidatus* Hepatoplasma crinochetorum and *Candidatus* Hepatincola porcellionum dominated in *P. scaber* from Germany (Kiel and Köln) and Canada (Fig. 3B), and in *O. asellus* (Fig. 3C) and *T. rathkii* from Germany (Fig. 3D); only *Candidatus* Hepatoplasma crinochetorum but not *Candidatus* Hepatincola porcellionum are prevalent in French *O. asellus* population Oa-4 (Fig. 3C), German isopods populations of *P. muscorum*, *A. vulgare*, and Canadian *A. perconvexus* (Fig. 3D). Taken together, it is obvious that symbiotic associations between these two bacteria and their isopod hosts are universal among terrestrial isopods. Besides these two symbiotic hepatopancreatic bacteria, γ -Proteobacteria and rod-shaped bacteria were observed in a small fraction of German population of *O. asellus* (14.3%) and Canadian population of *A. perconvexus* (6.3%), respectively, suggesting some diversity of hepatopancreatic symbionts in different isopod species and localities.

Since there are no symbiotic bacteria in the hepatopancreas of the marine isopod, *Idotea balthica* (Wang *et al.*, 2004c), *I. wosnesenskii* and *Gnorimosphaeroma oregonense* (Zimmer *et al.*, 2001), and since *Candidatus* Hepatincola porcellionum and *Candidatus* Hepatoplasma crinochetorum were not found in the semi-terrestrial isopod, *Ligia oceanica* (Wang *et al.*, 2004c) and *Ligia pallasii* (Yongjie Wang and Martin Zimmer, unpubl.), the widespread distribution of these two symbiotic bacteria in terrestrial isopod indicates that these hepatopancreatic symbionts occurred after colonization of land by an ancestor of recent isopods. Subsequently, the hepatopancreatic symbionts horizontally transferred among different species. It could be that these two symbiotic

types of hepatopancreatic bacteria infected some isopod ancestors occasionally. However, maintenance of the stable symbiont-host association possibly depends on both physiological requirement of isopod hosts, and availability as well as infection capability of symbiotic bacteria in the environment.

The significance of the isopod-microbe symbiosis remains unclear, while a nutrition basis of this symbiotic association was proposed (Zimmer and Topp, 1998) like for microbe-termite symbiosis (Breznak and Brune, 1994; Breznak, 2000; Brune and Friedrich, 2000; Brune, 2003; Buchner, 1965; Douglas, 1998). Molecular phylogenetic analyses of the symbiotic bacteria did not provide valuable insights to support previous speculations (Wang *et al.*, 2004a; Wang *et al.*, 2004b; Wang *et al.*, 2004c). Additionally, the geographical distribution of the symbionts revealed that the frequency of aposymbiotic individuals was high up to 80% in several isopod populations, suggesting facultative but not obligate interactions between symbionts and isopod hosts. Consequently, bacterial contributions to digestion of leaf litter by isopod seem unlikely. The underlying symbiosis mechanism needs to be explored in future work.

6.5.2 Cell density of hepatopancreatic symbionts of isopods

The cell density of *Candidatus* Hepatincola porcellionum and *Candidatus* Hepatoplasma crinochetorum symbionts varied (10^4 – 10^9) markedly within and between isopod populations (Fig. 4). Why and how do the isopod individuals harbour different cell numbers of similar symbiotic bacteria? One scenario proposed to explain this is that the isopod host regulates bacterial cell density in response to its own physiological requirement and/or environmental factors. For example, symbiont numbers could be regulated by limited nutrient availability from hosts, by factors manifested by the host that control the bacteria cell proliferation, etc. The underlying mechanism remains to be addressed.

6.5.3 Seasonal occurrence of hepatopancreatic symbionts

As a whole, however, although the occurrence varied over time, it did not display distinct seasonal trends, indicating that ecological factors, e.g., temperature shifts and food availability, do not directly and significantly affect the distribution of the

symbionts. However, high densities of symbiotic bacteria tend to occur in summer and autumn (Fig. 5B), revealing that environmental factors, e.g., temperature and food, could somehow indirectly influence the bacterial density. During winter, isopods show little metabolic actions. Consequently, hepatopancreatic bacteria possibly cease growing and even decline due to limited nutrient supply from their host; on the other hand, the low temperature could also inhibit directly bacterial growth and lead to the reduction of bacterial cell. In spring, low temperature might still restrict the growth of hepatopancreatic bacteria. By contrast, high temperature and good nourishment availability during summer and autumn could lead to rapid growth of bacterial symbionts.

In conclusion, our study clearly demonstrates that *Candidatus* Hepatincola porcellionum and *Candidatus* Hepatoplama crinochetorum in the hepatopancreas of isopod hosts are widespread in geographically distant isopod populations, and represent the prevalent symbionts in terrestrial isopod, which indicates either coevolution of isopod-symbiont or horizontal transmission of these symbionts among isopod species. Symbiont-free individuals as well as individuals harbouring only low numbers of the bacterial symbionts do not support an obligate mutualistic relationship between isopod host and hepatopancreatic symbionts. Work on comparison of congruent evolution between isopod and symbiont as well as the symbiont transmission in isopod progeny will further give insights into the symbiotic evolution and association.

6.6 References

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7 Environmental transmission of hepatopancreatic bacterial symbionts in the terrestrial isopod *Porcellio scaber* (Crustacea: Isopoda)

7.1 Abstract

The terrestrial isopod *Porcellio scaber* harbors *Mycoplasma*-like symbiotic bacteria, the candidate species *Hepatoplasma crinochetorum*, in its hepatopancreas. This study investigated the mode by which these bacterial symbionts are acquired by offspring. Mother isopods and their juveniles were maintained under unsterile conditions (control group) and sterile conditions of reduced external contamination of these symbionts (treatment group), respectively. Based on specific fluorescence-labelled oligonucleotide probe hybridization (whole-cell and thin section) and diagnostic PCR techniques, bacterial symbionts were detected in early developmental stages of juveniles from control group, but neither in embryos from both groups nor in early developmental stages of juveniles from the treatment group. Our data suggest that hepatopancreatic symbionts of *P. scaber* are environmentally transmitted to the new progeny.

Keywords: Crustacean; Isopod; hepatopancreatic symbionts; transmission

7.2 Introduction

Many terrestrial arthropods have evolved perfect transmission mechanisms to ensure that progeny are inoculated specific bacterial symbionts (Cary and Giovannoni, 1993). Three pathways have been proposed to classify the transmission of the symbiotic bacteria in invertebrates (Cary and Giovannoni, 1993): (i) vertical transmission, with symbionts in or on the gametes of the female parent and being transferred to offspring via host reproduction, as in many bacteriocyte-insect symbioses, or symbionts being transmitted to progeny through mother's gland secretions (Aksoy, 2003); (ii) horizontal transmission, symbionts spreading between contemporary hosts; (iii) environmental transmission, the new host generation being reinfected by symbionts occurring as free-living form in host environment (McFall-Ngai, 2002).

In previous study, we identified hepatopancreatic symbionts in the terrestrial isopod *Porcellio scaber* (Crustacea: Isopoda) as *Rickettsia*-like and *Mycoplasma*-like bacteria (Wang *et al.*, 2004a; Wang *et al.*, 2004b). Subsequently, we found that the *Mycoplasma*-like symbionts are widespread in geographically distant populations of 6 different terrestrial isopod species (Wang *et al.*). In light of these data, we proposed that horizontal and/or environment transmission of these symbionts possibly occurred in terrestrial isopods. Knowledge of mechanisms of symbiont transmission is critical to our understanding of symbiotic association of isopod hosts-hepatopancreatic bacteria as well as evolution and dispersal of isopods.

In this study, we checked the presence of *Mycoplasma*-like symbionts in embryo and two early stages of juveniles of *P. scaber* using molecular techniques in two groups of isopod mothers cultivated in unsterilized conditions vs. sterilized conditions to elucidate the mode of symbiont transmission from generation to generation.

7.3 Materials and Methods

7.3.1 Experiment design

Gravid females of *Porcellio scaber* were collected in decaying wood near the botanical garden of the University of Kiel, Germany. They were divided into treatment and control groups. In the treatment group, isopods were washed 5 times in autoclaved water, surface-sterilised with 70% ethanol and UV light ($\lambda = 254$ nm) for 10 sec and 5 min, respectively, and washed 5 times in water again in order to exclude an external contamination with hepatopancreatic symbiotic bacteria. Subsequently, they were maintained individually in sterile Petri dishes (15 × 85 mm) with six pieces of sterile filter paper (80 mm) on the base. The filter paper was wetted using filtered ($\text{\O} 0.45 \mu\text{m}$) supernatant water extracted from soil and renewed in every two days during the first two weeks. Autoclaved leaf litter (*Rubus fruticosus*) was provided as food. Gravid females in the control group were also kept individually in Petri dishes, but were not subject to surface-sterilising treatment, and fed on unsterilised leaf litter. Unfiltered supernatant water extracted from soil was used to moisten the filter paper. All isopods were maintained at 15°C, 16 h light, 8 h dark to harvest embryos before hatching and juveniles after release from their mother's brood pouch at day 1 (stage 1) and at day 7 (stage 2). For each group, 30 embryos or 40 juveniles (stage 1, 20; stage 2, 20) were collected from 10 female each (Table 1). Mother isopods were dissected immediately after obtaining their embryos or juveniles to check for hepatopancreatic symbionts.

Table 1 Number of gravid female isopods and their embryos and juveniles examined, and detecting methods for *Mycoplasma*-like symbionts

Detecting method	Mother	Control group			Treatment group		
		10	10		10	10	
		Embryo	Juvenile 1	Juvenile 2	Embryo	Juvenile 1	Juvenile 2
Whole-cell hybridisation	40	10 × 10	–	–	10 × 10	–	–
Diagnostic PCR	2	10 × 10	10 × 10	10 × 10	10 × 10	10 × 10	10 × 10
Thin section hybridisation	–	10 × 10	10 × 10	10 × 10	10 × 10	10 × 10	10 × 10

To both control and treatment groups, twenty individual mothers harbouring *Mycoplasma*-like symbionts were selected out of 34 and 39 females, respectively. Subsequently, their embryos and juveniles were subjected to analysis of the occurrence of this type of symbionts (Table 1).

7.3.2 DNA extraction and PCR amplification

In both control and treatment groups, mothers and juveniles were surface-sterilized as above. Subsequently, the juveniles and dissected embryos and hepatopancreas of the mothers were immediately frozen and stored at -20°C . DNA extraction followed the protocol provided by DNeasy[®] Tissue kit (Qiagen). The purified DNA was eluted in 100 μl of AE buffer provided in the kit.

The 16S rRNA genes of the candidate species *Hepatoplasma crinochetorum* were detected using the specific forward primer PsHc372f (5'-CAG CAG TAG GGA ATT TTT CAC-3') (Wang *et al.*) and the bacteria-specific reverse primer 1492r (Weisburg *et al.*, 1991). PCR amplification was performed in a 200- μl PCR tube filled with 50- μl reaction volume containing 200 μM of each dNTP, 5.0 μl of a 10 \times 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 . Aliquots of 5 μl of the PCR products were resolved in 1.0% (wt/vol) agarose gels in 1.0 \times TAE buffer (40 mM Tris-acetate, 1mM EDTA). The gels were visualized under ultraviolet light after ethidium bromide staining (0.5 $\mu\text{g}/\text{ml}$).

7.3.3 DAPI staining and Fluorescence in situ hybridization

Embryos and midgut glands were thoroughly homogenized in 500 μl PBS, and formaldehyde was added to a final concentration of 4% (wt/vol). After fixation for 14 h at 4°C , the samples were centrifuged at $10,000 \times g$ for 5 min. Pellets were washed three times in PBS, finally resuspended in 250 μl PBS plus 250 μl ethanol (100%), and stored at -20°C until analysis.

Samples were filtered onto polycarbonate filters (0.2 µm pore size) and dried at 46°C for 30 min. Samples were stained with DAPI (4',6-diamidino-2-phenylindole) and hybridized with fluorescently labelled oligonucleotides as described by (Wagner *et al.*, 1993); negative controls with an EUB338 antisense probe (5'-ACTCCTACGGGAGGCAGC-3') (Wallner *et al.*, 1993) were used to exclude non-specific 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 1,000-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 as previously described (Dubilier *et al.*, 1995). Samples were hybridized with the fluorescein-labeled Bacteria-specific probe EUB338 (5'-GCTGCCTCCCGTAGGAGT-3') (Amann *et al.*, 1990) and the Cy3-labeled sequence-specific probe PsSym352 as described in (Wang *et al.*, 2004a); negative controls were included to exclude non-specific binding.

7.4 Results

7.4.1 Whole-cell hybridisation

No symbiotic bacteria were detected in the homogenates of 100 embryos obtained from 10 mothers in both control and treatment groups, respectively, which corroborate the results of both specific PCR and thin section hybridisation detection (see below).

7.4.2 PCR analysis

PCR products were not obtained from DNA samples extracted from embryos in both control and treatment groups nor from juveniles in treatment group (Fig. 1). By contrast, an expected single band of 1,162 bp was detected from DNA samples extracted from juveniles in the control group (Fig. 1), which indicates the presence of the candidate species *Hepatoplasma crinochetorum*.

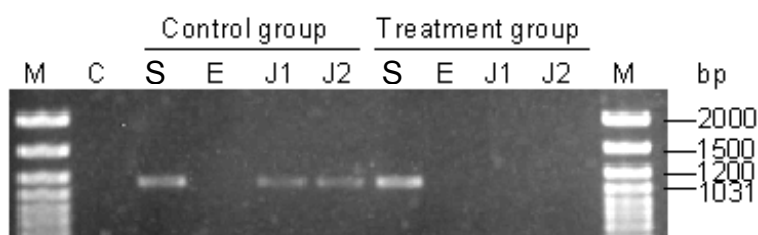


Fig. 1. PCR detection of the candidate species *Hepatoplasma crinochetorum*, in adult female's embryos and juveniles of *Porcellio scaber*. Lanes: M, DNA marker; C, negative control; S, E, J1 and J2 in both control and treatment groups represent the *Mycoplasma*-like symbionts in mother, embryo, juveniles at day 1 and day 7, respectively.

7.4.3 Thin section hybridisation analysis

Data from whole-cell hybridisation and PCR were further confirmed by the observation of thin sections of whole embryos and juveniles in both control and treatment groups. As shown in Figure 2, distinguished signals were not observed in embryos from both groups, nor in the hepatopancreas and other sections of juveniles from treatment group, but were detected in the hepatopancreas of juveniles from the control group. However, infection rates of the juveniles in both stages 1 and 2 were low and varied among different sibships (Table 2).

Table 2 Infection rates of *Porcellio scaber* juveniles by the candidate species *Hepatoplasma crinochetorum* in the control group using thin section hybridisation analysis

		Mother No.									
		1	2	3	4	5	6	7	8	9	10
Infection rate (%)	Stage 1	0	0	10	10	0	0	20	0	10	0
	Stage 2	0	10	20	10	0	0	20	20	0	0

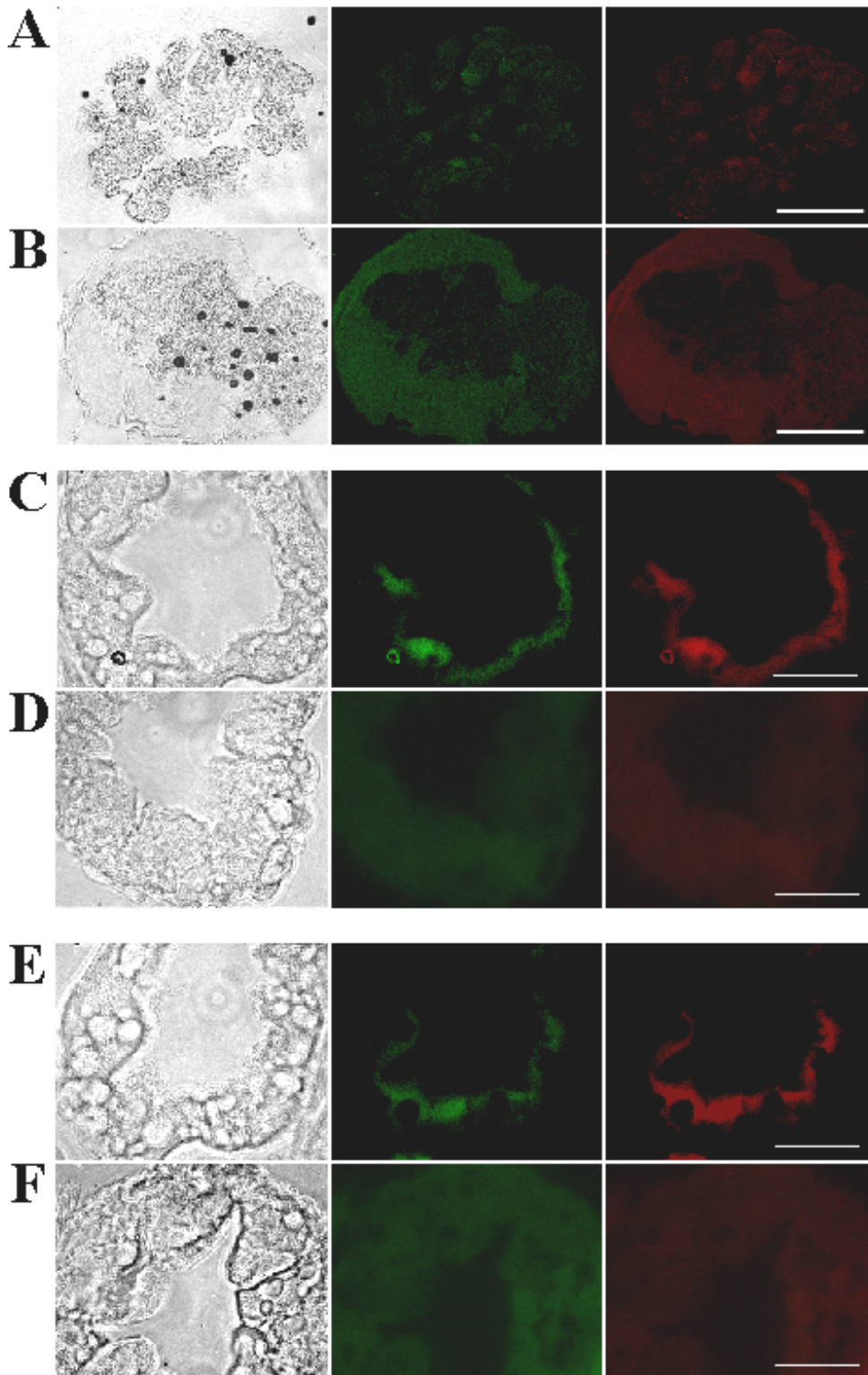


Fig. 2. Thin section hybridisation with specific fluorescence-labelled oligonucleotide probes to detect the candidate species *Hepatoplasma crinochetorum*. Black-white

panel, phase-contrast image; green colour, hybridisation with probe EUB338 specific for Eubacteria; red colour, hybridisation with probe PsSym352 for the symbiotic bacteria. A, C and E, embryo, juvenile at day 1 and day 7 in the control group; B, D and F, embryo, juvenile at day 1 and day 7 in the treatment group. Scale bar, 50 μm in A and B; 5 μm in C-F.

7.5 Discussion

In the present study, the environmental transmission mode of the hepatopancreatic symbiont of the terrestrial isopod *Porcellio scaber*, the candidate species *Hepatoplasma crinochetorum*, was demonstrated by using molecular techniques.

The lack of bacterial symbionts in embryos in both control and treatment groups indicate that symbiotic bacteria are neither transmitted directly from mother to offspring as is known from many strictly mutualistic symbiotic associations, e.g., in insects with a single diet (Douglas, 1998), in some marine worms and bivalves (Sipe *et al.*, 2000), nor were transferred to eggs or hatched embryos in the brood pouch by invading the marsupium from their environments.

The presence of symbionts in juveniles of the control group, but not in that of the treatment group, strongly suggests that the transmission of symbionts occurs via a free-living symbiont form inhabiting in the soils and/or leaf litter. Conceivably, only a single bacterium would be necessary to initiate the symbiosis in a host juvenile. Detection at this level of resolution with respect to the soil and leaf litter samples is not yet possible with molecular approaches, e.g., PCR and FISH, used in this study. Faeces from the isopod mother carrying symbionts were not removed from the Petri dishes in both groups throughout the experiment. Accordingly, if juveniles in control group were infected by the symbionts in faeces of their mothers, these bacteria should also occur in the hepatopancreas of juveniles in the treatment group, but we did not find them. Consequently, symbiont transmission initiating faeces by isopod hosts seems to be unlikely.

The presence of symbionts in the early stage 1 juveniles suggests that the acquirement of symbionts occurred once the juveniles were exposed to their environment. The slightly higher infection rates in stage 2 reveal that the infection possibility occurred several times during juvenile development.

All juveniles from 4 out of 10 tested sibships in the control group were aposymbiotic, and juveniles from the other 6 sibships revealed only low infection rates. By contrast, in natural *P. scaber* populations >40% of adult individual isopods bear such symbiotic bacteria (Wang *et al.*). Thus, the low incidence of symbionts in laboratory populations possibly results from the limited access to, and insufficient reservoir of, free-living potential symbionts provided in the experiment compared with the natural environments. However, the low recruitment rate by the new progeny also suggests the optional association in isopod-*Candidatus* Hepatoplasma crinochetorum symbiosis.

Possible transmission routes are proposed as shown in Figure 3. The symbionts' affiliation with mycoplasma parasites and pathogens (Wang *et al.*, 2004a) might hint on their ability to penetrate the very soft and thin cuticle of the early stage of juveniles and reach the hepatopancreas via the hemolymph.

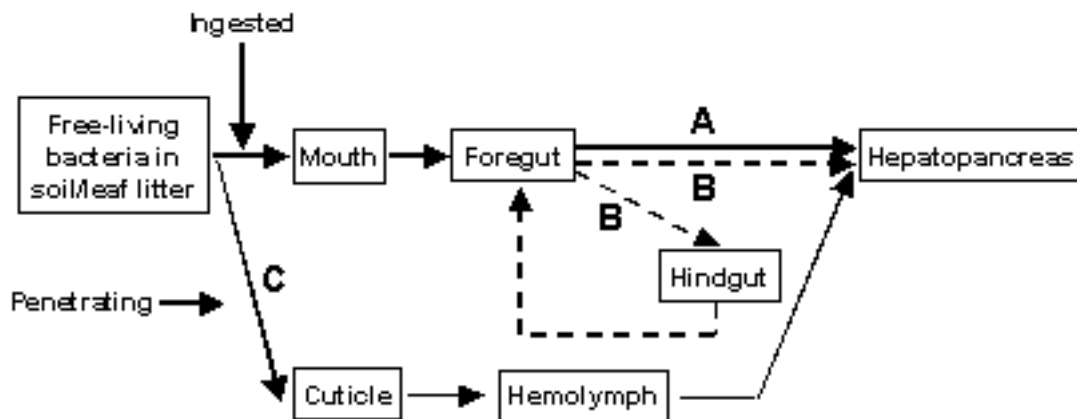


Fig. 3. Model of potential transmission routes in the isopod-Hepatoplasma symbiosis. Free-living forms of potentially symbiotic bacteria enter the isopod mouth through feeding. After passing the foregut, they may enter the Hepatopancreas directly (A) or by passing hindgut and backing to foregut (B). They might also invade hepatopancreas of early juvenile by penetrating the cuticle and passing by hemolymph (C).

The recruitment of this specific symbiont from the soil habitats housing huge numbers of different microorganisms seem to employ some complex recognition mechanisms between the isopod host and the free-living potential symbionts, perhaps similar to those which occur between root-nodulating, nitrogen-fixing *Rhizobium* species and legumes, which have represented the principal model for Prokaryotic-eukaryotic symbioses (Frayse *et al.*, 2003). Further investigations will be necessary to identify these recognition mechanisms and to understand how host-symbiont relationships are unique and ubiquitous in geographic range.

7.6 References

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8 General summary and conclusions

This study focuses on the hepatopancreatic bacterial symbionts in crustacean isopods, which originated from marine environments but successfully colonized terrestrial and freshwater habitats during evolution. The studies presented here revealed phylogenetic affiliations of bacterial symbionts in different isopod species collected from diverse habitats and provided insights into the evolution and associations of hepatopancreatic bacterium-isopod host symbiosis.

8.1 Phylogenetic identification of hepatopancreatic symbionts

8.1.1 Hepatopancreatic symbionts in the cosmopolitan terrestrial isopod *Porcellio scaber*

DAPI-stained homogenates of the midgut glands confirmed a dense bacterial colonization ($>10^6$ cells per individual). In some individuals, the cells were rod-shaped (1.5–3.8 $\mu\text{m} \times 0.5 \mu\text{m}$) and moderately to strongly curved; in other individuals, they had a spherical shape and fell into two size classes with diameters of 0.2–0.3 μm (“small spheres”) and 0.5–0.8 μm (“large spheres”). Based on comparative sequence analysis and fluorescence-labelled oligonucleotide probes in situ hybridisation of their 16S rRNA genes, the rod-shaped bacterial symbionts represent a novel lineage in the α -subclass of Proteobacteria, and their closest (albeit distant) relatives ($<81\%$ sequence similarity) are among the bacterial order of Rickettsiales, which are intracellular symbionts or pathogens of animals; the sphere-shaped bacterial symbionts represent a novel lineage among the bacterial class of Mollicutes, only distantly related ($<82\%$ sequence identity) to members of the Mycoplasmatales and Entomoplasmatales, which contain parasites and pathogens of animals.

The Rickettsiales comprise five major genogroups, namely Rickettsiae, Ehrlichiae, Neorickettsiae, Wolbachiae and Holosporae (Fredricks, 2001), which contain bacteria

living as pathogens, parasites or symbionts within the cytoplasm or nucleus of eukaryotic cells. In contrast to these lineages, the *Rickettsia*-like bacteria colonizing the hepatopancreas of *P. scaber* are extracellular symbionts. Presently, the Mollicutes are considered to consist of five separate phylogenetic clades: the *Asteroleplasma* group, the *Acholeplasma*–*Anaeroplasma* group, the *Mycoplasma hominis* group, the *Mycoplasma pneumoniae* group, and the *Spiroplasma* group (Razin, 2000). All phylogenetic analyses supported that the hepatopancreatic symbionts of *P. scaber* represent a sixth lineage among the Mollicutes. Although Mollicutes are widespread commensals or pathogens of plants, humans, mammals, reptiles, fish, and also of other arthropods (Razin *et al.*, 1998), little is known about their occurrence in crustaceans. This is the first study demonstrating the colonization of the hepatopancreatic lumen of a crustacean host by extracellular *Mycoplasma*-like symbionts.

Both the *Rickettsia*-like and *Mycoplasma*-like symbionts possess a stalk-like appendage. In many cases, the stalks were in contact with the microvillous brush border of gland epithelium, often inserted into the space between the microvilli. Although adhesion of microorganisms to epithelial surfaces is characteristic for intestinal habitats, the occurrence of stalk-like structures that insert between the microvilli of the epithelial brush border is rather unusual among bacterial symbionts of invertebrates, and may be a case of convergent evolution, driven by the same functional necessities. Whether the stalk functions in the prevention of a complete washout of the symbionts into the digestive tract together with the hepatopancreatic secretions, or in cross-talk between symbionts and their hosts, remains open to speculation and further study.

Based on the isolated phylogenetic position and unique cytological properties, the provisional names ‘*Candidatus Hepatincola porcellionum*’ and ‘*Candidatus Hepatoplasma crinochetorum*’ were proposed to classify these two new taxon of Rickettsiales and Mollicutes, respectively, colonizing the hepatopancreas of *P. scaber*.

8.1.2 Hepatopancreatic symbionts in other isopod species

8.1.2.1 The terrestrial isopod *Onicus asellus*

Spherical as well as curved rod-shaped bacteria were detected in the hepatopancreas of the terrestrial isopod *Onicus asellus*, which morphologically correspond to the symbionts found in the terrestrial isopod *Porcellio scaber*. Based on comparative sequence analysis and fluorescence-labelled oligonucleotide probes in situ hybridisation of their 16S rRNA genes, the sphere-shaped bacterial symbionts represent a novel lineage among the bacterial class of Mollicutes, only distantly related (<81% sequence identity) to members of the Mycoplasmatales and Entomoplasmatales, but give high (>97%) sequence identity to the 16S rRNA genes retrieved from the sphere-shaped, *Mycoplasma*-like hepatopancreatic symbiont, harboured by *P. scaber*, which indicates that they are intimate lineage members; based on specific probe in situ hybridisation and denaturing gradient gel electrophoresis (DGGE) analyses, the curved rod-shaped bacterial symbionts are closely related to the *Rickettsia*-like symbionts identified from *P. scaber*.

8.1.2.2 The semi-terrestrial isopod *Ligia oceanica*

Based on comparative sequence analysis and fluorescence-labelled oligonucleotide probes in situ hybridisation of their 16S rRNA genes, the rod-shaped bacterial symbionts observed in the hepatopancreas of the semi-terrestrial isopod *Ligia oceanica* give very high sequence identity (>99%) to the members in the bacterial genus *Pseudomonas*, γ -Proteobacteria.

8.1.2.3 The marine isopod *Idotea balthica*

DAPI-staining, EUB338-specific hybridisation, and PCR-amplification of eubacterial DNA revealed that there are no hepatopancreatic bacteria in the marine isopod *Idotea balthica*.

8.1.2.4 The freshwater isopod *Asellus aquaticus*

Phylogenetic analyses of bacterial 16S rRNA genes demonstrated that hepatopancreatic symbionts in the freshwater isopod, *Asellus aquaticus*, occurred in high diversity. They were closely related to members of the bacterial genera *Rhodobacter* (>95% sequence identity), α -Proteobacteria, *Burkholderia* (>99% sequence identity), β -Proteobacteria, *Aeromonas* (>99% sequence identity), γ -Proteobacteria, and *Rickettsiella* (>96% sequence identity), γ -Proteobacteria.

8.2 Geographical distribution of hepatopancreatic symbionts

According to results obtained from a combination of specific fluorescence-labelled oligonucleotide probe hybridisation and diagnostic PCR techniques, the *Rickettsia*-like and *Mycoplasma*-like symbionts represent the major symbiotic bacteria in the terrestrial isopods examined. Both of them were found in all of 6 *P. scaber* populations (n=177) collected from geographically distant localities, Germany (Kiel and Köln) and Canada (Bamfield, Vancouver Island and Haines Island), in 3 *O. asellus* populations (n=60) sampled in geographically distant sites in Germany (Kiel and Köln), and in *Trachelipus rathkii* from Kiel. In addition, the *Mycoplasma*-like was also detected in French *O. asellus*, in German *Philoscia muscorum* and *Armadillidium vulgare*, and in Canadian *Alloniscus perconvesus*. Although their infection frequency varied within and between isopod populations, the *Mycoplasma*-like symbionts were overall more prevalent than the *Rickettsia*-like symbionts. Aposymbiotic isopod individuals were found in all of the six isopod species investigated with different frequency between populations.

8.3 Transmission of hepatopancreatic bacterial symbionts

Based on specific fluorescence-labeled oligonucleotide probe hybridization (whole-cell and thin section) and diagnostic PCR techniques, bacterial symbionts were detected in early developmental stages of juveniles from control group (without sterile treatments), but neither in early developmental stages of juveniles from the treatment group (with sterile treatments) nor in embryos from both groups. Our data suggest that

hepatopancreatic symbionts of *P. scaber* are environmentally transmitted to the progeny.

8.4 Conclusions

8.4.1 Evolution of symbiosis

Both the *Rickettsia*-like and *Mycoplasma*-like symbionts suggest a parasitic origin of recent hepatopancreatic symbionts.

No hepatopancreatic symbionts in marine isopods, but high number of them in semi-terrestrial, terrestrial, and freshwater isopods examined suggest that the occurrence of symbionts is closely related to isopod evolution towards terrestrial and freshwater habitats as well as nutritional shift from marine algae to leaf litter.

Symbiotic bacteria colonizing semi-terrestrial, terrestrial, and freshwater isopods belong to different phylogenetic affiliations, suggesting that they possibly underwent an evolutionary exchange or represent independent origins of symbiotic associations.

The diverse hepatopancreatic symbionts in the freshwater isopod, *A. aquaticus*, were possibly acquired several times independently and exchanged or lost after isopod hosts colonized and adapted to freshwater habitats.

The widespread *Rickettsia*-like and *Mycoplasma*-like symbionts in geographically distinct populations of different terrestrial isopod species (*P. scaber*, *O. asellus*, *Philoscia muscorum*, *Armadilidium vulgare*, *Trachelipus rathkii*, and *Alloniscus perconvexus*) suggest a horizontal transmission of them among terrestrial isopod during the evolution.

8.4.2 Symbiotic associations

Enzymes involved in degradation of cellulose, xylan and lignin in leaf litter and wood were found only in close relatives of the *Pseudomonas* sp. symbiont in the semi-terrestrial isopod *L. oceanica*, and of the *Aeromonas* sp. and *Burkholderia* sp. symbionts in the freshwater isopod, but not in close relatives of the *Rickettsia*-like and

Mycoplasma-like symbionts in terrestrial isopods. These data render the previous speculations on contributions of symbiotic bacteria to the digestion of terrestrial food sources (leaf litter) by isopod hosts less likely.

In addition, the occurrence of aposymbiotic isopod individuals, as well as the low infection rates (<20%) of isopod progeny by the wide spread *Mycoplasma*-like symbiont, suggest the lack of an obligate mutualistic symbiotic association between hepatopancreatic symbionts and isopod hosts. Parasitic or facultative mutualistic symbiotic relationship might be possible.

8.5 References

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9 Summary

Terrestrial isopods harbor uncultured bacterial symbionts in their midgut glands (hepatopancreas) that may have facilitated the evolutionary shift to utilizing terrestrial leaf litter during the course of colonizing land. In a first molecular-biological approach, we screened isopods from marine, semi-terrestrial, terrestrial, and freshwater habitats for hepatopancreatic bacterial symbionts by means of 16S rRNA gene cloning and sequencing, denaturing gel gradient electrophoresis (DGGE), fluorescence-labeled specific oligonucleotide probe *in situ* hybridization (FISH), and electron microscopy.

There are no hepatopancreatic bacteria in the marine isopod *Idotea balthica*, while high numbers of nearly uniform morphotypes of bacteria were detected in semi-terrestrial (*Ligia oceanica*), terrestrial (*Porcellio scaber*, *Oniscus asellus*, *Philoscia muscorum*, *Armadilidium vulgare*, *Trachelipus rathkii*, and *Alloniscus perconvesus*), and freshwater (*Asellus aquaticus*) isopods. Phylogenetic analyses demonstrated that the symbiotic bacteria in this three types of isopod species are not closely related but belong to bacterial taxa as different as *Pseudomonas* (γ -Proteobacteria, similarity >98%) in *L. oceanica*, Rickettsiales (α -Proteobacteria, similarity <81%) and Mycoplasmatales (Mollicutes, similarity <82%) in *P. scaber* and *O. asellus*, and *Rhodobacter* (α -Proteobacteria, similarity >95%), *Burkholderia* (β -Proteobacteria, similarity >98%), *Aeromonas* (γ -Proteobacteria, similarity >99%) and *Rickettsiella* (γ -Proteobacteria, similarity >96%) in *A. aquaticus*, respectively. These findings suggest that hepatopancreatic symbionts either have been acquired several times independently or have been exchanged during isopod evolution.

Rickettsia-like and *Mycoplasma*-like symbionts of terrestrial isopod represent prevalent symbionts in several geographically distinct isopod populations. These results suggest either a common ancestor of terrestrial isopod that already harboured these bacteria symbionts or horizontal inter-specific transmission of the symbionts. Based on FISH and diagnostic PCR techniques, our data suggest that the *Mycoplasma*-like hepatopancreatic symbionts of *P. scaber* are environmentally transmitted to the new progeny exposing to the external environment.

Except for the γ -Proteobacteria and β -Proteobacteria found in *L. oceanica* and *A. aquaticus*, respectively, close relatives of isopod symbionts are not known to produce any enzyme that might facilitate the digestion of leaf litter, suggesting that the proposed contribution of bacterial symbionts to digestive processes of isopod hosts is unlikely.

Zusammenfassung

Landasseln beherbergen in ihren Mitteldarmdrüsen (Hepatopankreas) unkultivierbare bakterielle Symbionten, die möglicherweise den evolutiven Wechsel zur Nutzung terrestrischer Laubstreu als Nahrung während der Landbesiedlung erleichtert haben. In einem ersten molekularbiologischen Ansatz untersuchten wir Asseln mariner, semi-terrestrischer, terrestrischer und limnischer Habitaten bezüglich der Existenz und Identität hepatopankreatischer bakterieller Symbionten mithilfe der Klonierung und Sequenzierung ihrer 16S rRNA-Gene, denaturierender Gradienten Gelelektrophorese (DGGE), *in situ*-Hybridisierung mit fluoreszenzmarkierten spezifischen Oligonukleotid-Sonden (FISH) und Elektronenmikroskopie.

In der marinen Art *Idotea balthica* waren keine hepatopankreatischen Bakterien nachweisbar, während hohe Dichten morphologisch ähnlicher Bakterien in semi-terrestrischen (*Ligia oceanica*), terrestrischen (*Porcellio scaber*, *Oniscus asellus*, *Philoscia muscorum*, *Armadillidium vulgare*, *Trachelipus rathkii* und *Alloniscus perconvexus*) und limnischen (*Asellus aquaticus*) Arten gefunden wurden. Phylogenetische Analysen zeigten, dass die symbiotischen Bakterien dieser drei Gruppen von Asselarten miteinander nicht nah verwandt sind, sondern zu so unterschiedlichen Taxa wie *Pseudomonas* (γ -Proteobacteria; >98% Sequenzähnlichkeit) in *L. oceanica*, Rickettsiales (α -Proteobacteria; <81% Ähnlichkeit) und Mycoplasmatales (Mollicutes; <82% Ähnlichkeit) in *P. scaber* und *O. asellus*, sowie *Rhodobacter* (α -Proteobacteria; >95% Ähnlichkeit), *Burkholderia* (β -Proteobacteria; >98 % Ähnlichkeit), *Aeromonas* (α -Proteobacteria; >99% Ähnlichkeit) und *Rickettsiella* (γ -Proteobacteria; >96% Ähnlichkeit) in *A. aquaticus*, gehören. Diese Befunde lassen vermuten, dass hepatopankreatische Symbionten entweder mehrmals unabhängig akquiriert oder während der evolutiven Aufspaltung der Isopoda ausgetauscht wurden.

Rickettsia-ähnliche und *Mycoplasma*-ähnliche Symbionten terrestrischer Asseln repräsentieren die dominanten Symbionten in verschiedenen geographisch distinkten Populationen unterschiedlicher Arten. Diese Befunde legen entweder einen gemeinsamen Vorfahren dieser Arten, der bereits diese Symbionten beherbergte, oder eine zwischenartliche horizontale Übertragung bakterieller Symbionten nahe. Unsere Ergebnisse aus FISH und diagnostischer PCR machen eine horizontale Übertragung der *Mycoplasma*-ähnlichen Symbionten auf Nachkommen wahrscheinlich.

Außer den γ -Proteobacteria und β -Proteobacteria in *L. oceanica* beziehungsweise *A. aquaticus* sind nahe Verwandte der bakteriellen Symbionten der untersuchten Asselarten nicht dafür bekannt, dass sie Enzyme produzieren, die die Verdauung von Laubstreu unterstützen. Folglich erscheint ein Beitrag hepatopankreatischer Symbionten zu Verdauungsprozessen unwahrscheinlich.

10 Outlook

1. Clone genes encoding cellulases from hepatopancreatic epithelial cells of isopod host to elucidate the origin of the cellulases detected in hepatopancreas.
2. Whole genome sequencing the *Mycoplasma*-like and *Rickettsia*-like symbionts to reveal comprehensive genome information for giving insights into the evolution, genotypic and phenotypic characteristics of the symbionts.
3. Cultivate the *Mycoplasma*-like and *Rickettsia*-like symbionts *in vitro* using the isolating methods for other members in Mycoplasmatales and Rickettiales.
4. Further survey the distribution of the *Mycoplasma*-like and *Rickettsia*-like symbionts in phylogenetic distant lines of isopod species colonizing marine and semi-terrestrial habitats to retrieve the evolution of the symbiosis.

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Wang, Yongjie. 2001. Bioremediation and biotreatment of specific environmental concern, p. 536-573: *In* S. Li et al. (eds), *Environmental Microbiology*. The High Education Press, Beijing.

Declaration

Hereby I declare that this dissertation was written independently by me and represents my own work. This work has not been submitted for any other degree or qualification. My thesis focuses on microbe-animal symbiosis.

Yongjie Wang

Kiel, 18.09.2004