Towards understanding a holobiont: Host-microbe interactions in *Hydra*

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Summary

Epithelia in all animals, which are exposed to the environment, are colonized by more or less diverse communities of microbes. Since *Hydra* is an early-branching metazoan and has preserved much of the genetic complexity of the common metazoan ancestor it promises to be highly informative to discover evolutionary conserved mechanisms controlling epithelial host-microbe interactions.

To obtain a better understanding of the microbiota associated with phylogenetically ancient epithelia, I identified the epibiotic and endosymbiotic bacteria of two species of the cnidarian *Hydra* on the basis of rRNA gene comparisons. I discovered that individuals from both species differ greatly in their bacterial microbiota. *Hydra oligactis* appears to be associated with only a limited number of microbes, while H*ydra vulgaris* polyps have a more diverse microbiota. Surprisingly, the microbial composition showed similar characteristics comparing polyps from culture maintained in the laboratory for more than 30 years with polyps from the wild. The significant differences in the microbial communities between the two species and the maintenance of specific microbial communities over long periods of time strongly indicate distinct selective pressures imposed on and within the epithelium.

To understand the factors involved in the maintenance of the specific association I first analysed how epithelial homeostasis affects the associated microbiota. I could show that eliminating derivates of the interstitial stem cell lineage from the epithelium causes significant changes in hydra´s microbial community. Due to the loss of the interstitial cell lineage, bacteria of the β-Proteobacteria group decreased and bacteria of the Bacteroidetes group increased in relative abundance. These results indicate a previously unrecognized link between cellular tissue composition and microbiota.

To elucidate the role of the innate immunity, especially the antimicrobial peptides (AMP), in shaping the commensal microbiota, I made use of transgenic *Hydra* overexpressing the AMP periculin1a. I could show that overexpression of an endogenous AMP not only reduces the bacterial burden but also leads to drastic changes in the biodiversity of the colonizing microbiota. These findings indicate that AMPs as an evolutionary old 'invention' may play a crucial role in shaping the composition of the commensal microbiota. Antimicrobial peptides, until now known as gene–encoded key elements of innate immunity, therefore, appear also to have an essential role in host-microbe co-evolution.

Zusammenfassung

Epithelien, die im Kontakt mit der Umwelt stehen, sind in der Regel mit einer sehr unterschiedlichen mikrobiellen Gemeinschaft besiedelt. Auf Grund der Tatsache, dass Hydra zu den basalen Metazoen gehört und eine ähnliche genetische Komplexität wie höhere Metazoen besitzt, ist es ein vielversprechendes Model für die Untersuchung der Kontrollmechanismen in epithelialen Wirt-Bakterien Interaktionen.

Um ein grundlegendes Verständnis für bakterielle Gemeinschaften zu erhalten, die phylogenetisch alte Epithelien besiedeln, identifizierte ich zunächst die epibiotischen und endosymbiotischen Bakterien von zwei verschiedenen Hydraarten. Ich fand heraus, dass sich die bakterielle Besiedlung zwischen den Arten signifikant voneinander unterscheidet. Polypen der Art *Hydra vulgaris* waren mit einer diverseren bakteriellen Gemeinschaft besiedelt als *Hydra oligactis* Polypen. Erstaunlicherweise ähnelten sich die bakteriellen Gemeinschaften von Individuen der gleichen Arten, unabhängig davon, ob Tiere aus dem Freiland oder aus einer 30jährigen Laborkultur untersucht wurden. Die signifikanten Unterschiede in der mikrobiellen Besiedlung zwischen den Arten und die dauerhaft aufrechterhaltene Assoziation innerhalb einer Art weisen auf einen starken Selektionsdruck im Epithel hin.

Um die Faktoren zu verstehen, die an der Aufrechterhaltung der spezifischen Assoziation beteiligt sind, analysierte ich als Erstes den Einfluss der epithelialen Homöostase auf die assoziierten Bakterien. Ich konnte zeigen, dass das Verschwinden der interstitiellen Zelllinie einen signifikanten Effekt auf die mikrobielle Gemeinschaft ausübt. Dieses Ergebnis weist auf eine bisher nicht gekannte Verbindung zwischen der zellulären Gewebezusammensetzung im Epithel des Wirtes und der assoziierten mikrobiellen Gemeinschaft hin.

Als ein weiterer Faktor wurde der Einfluss des angeborenen Immunsystems, im speziellen der antimikrobiellen Peptide (AMP), auf die mikrobielle Besiedlung untersucht. Durch die Überexpression des AMPs periculin1a in ektodermalen Epithelzellen konnte ich zeigen, dass nicht nur die Anzahl der Bakterien stark reduziert wurde, sondern sich auch die Zusammensetzung der bakteriellen Gemeinschaft signifikant veränderte. Dieses Ergebnis zeigt, dass AMPs zusätzlich zur Pathogenabwehr eine zentrale Rolle in der Kontrolle der kommensalen Mikrobiota spielen und somit essentielle Faktoren in der Koevolution von Wirt und Bakterien sind.

General Introduction

The concept of the 'superorganism' and the holobiont theory

In the last decades animal–microbe interactions and the resulting co-evolution are considered as significant drivers of animal evolution and diversification. Thus, the question on which level selection takes place is still an ongoing debate in the theory of evolution. In the traditional view of evolutionary biologists adaptation takes place on the level of an individual or a gene, known as the concept of individual selection. Wilson & Sober expanded this concept to the model of a 'superorganism' which considers the selection on individuals (or genes), but additionally also on single- or multi-species communities (Wilson and Sober, 1989). In this model superorganisms are considered as a unit of functional organisation. The best studied example for the evolution of a superorganism is the eukaryotic cell. The prokaryotic character of mitochondria and chloroplasts suggest that they probably derived from endocytoesd bacteria (Gray et al., 1999; Gray and Doolittle, 1982; Yang et al., 1985).

Considering the concept of the 'superorganism', Rosenberg and colleagues in 2006 published the 'coral probiotic hypothesis' (Reshef et al., 2006). They assume that corals are able to adapt rapidly to changing environmental conditions by altering their associated microbiota and therefore, would be able to adapt much faster than by mutation and selection. They support their hypothesis by three observations: (i) corals are associated with diverse microbiota (Bourne and Munn, 2005; Rohwer et al., 2002), (ii) the associated microbiota change in response to environmental stress, as diseases (Pantos et al., 2003; Ritchie and Smith, 1995) or seasons (Koren and Rosenberg, 2006) and (iii) corals are able to develop resistance against pathogens although they lack adaptive immune response (Reshef et al., 2006). Referred to this case the authors suggest that changed microbiota provide antimicrobial substances that help the coral host to develop resistance against the pathogens (Ritchie, 2006).

Combining the 'coral probiotic hypothesis' and the concept of 'superorganisms' the holobiont theory was created which unites the host organism and all of its associated microbes (Rosenberg et al., 2007; Zilber-Rosenberg and Rosenberg, 2008). The authors support their hypothesis by the following arguments: (i) there has never been any animal or plant free of microorganisms, (ii) the holobiont has its own specific properties that are not necessarily the

sum of those of the host and its microbiota (iii) holobionts have their specific structures, (iv) each holobiont exists by itself, facing its environment, competing as a consortium with other holobionts and (v) the hologenome is transmitted from one generation to the next with high accuracy (Zilber-Rosenberg and Rosenberg, 2008). Therefore, the hologenome combines genetic information of the host organism and its associated microbes (Zilber-Rosenberg and Rosenberg, 2008).

Each holobiont is a dynamic assemblage of the host and its associated microbes, therefore the hologenome should be considered as the unit of natural selection in evolution. Depending on the variety of different niches provided by the host, which can change with the developmental stage, the diet or other environmental factors, a more or less diverse microbial community can establish with a given host species (Zilber-Rosenberg and Rosenberg, 2008). In many cases the number of associated microbes and their combined genetic information far exceed the genetic information of their host (Backhed et al., 2005; Zilber-Rosenberg and Rosenberg, 2008) and therefore, have a major impact on the holobiont. To maintain the holobiont between generations the accuracy of transmission is essential. Two major categories are known for the transmission of bacteria from one to another generation: (i) transovarian and (ii) environmental transmission (McFall-Ngai, 2002), while many intermediate modes are existing. In many cases the mechanisms underling the modes of transmission and the maintenance of the holobiont over generations are not well understood.

Epithelial host-microbe interactions

The diversity of microbes colonizing host epithelia is a result of co-evolution between host and the according microbial community. Molecular analysis of the bacterial microbiota in different mammalian guts uncovered an unprecedented bacterial diversity which is specific for each host (Ley et al., 2008), indicating that bacterial communities coevolved with their vertebrate hosts. This was also nicely demonstrated in a reciprocal transplantation experiment of the microbial community of mice and zebrafish. From this experiment, first evidence was provided (Rawls et al., 2006) that the gut epithelium is actively shaping the microbiota.

Gordon and colleagues (Ley et al., 2006) suggest that the specific structure of the microbial community associated with a given host is a result of natural selection at two levels. First, competition between members of the microbiota would exert “bottom up” selection and

second, the host level would represent a "top-down" selection on the microbial community (Ley et al., 2006). The innate immune system as the host's first line of contact with the microbiota is expected to play a crucial role in this "top-down" selection of microbiota. Supporting this view, impaired host innate immune responses can be linked directly to disturbances in composition of the microbiota and ultimately to human disease. Crohn's disease and ulcerative colitis patients, for example, have abnormal gastrointestinal microbiota characterized by the depletion of members of the phyla Firmicutes and Bacteroidetes (Frank et al., 2007), two bacterial divisions dominating the distal gut microbiota (Ley et al., 2006; Rawls et al., 2006). Interestingly, patients with Crohn's disease also show a reduced antibacterial activity in their intestinal mucosal extracts with strongly reduced expression of paneth cell α-defensins compared to a control group of patients (Wehkamp et al., 2005). Additionally, defects in key signal transmitters of innate immunity such as MyD88 affect the composition of the colonizing microbiota (Wen et al., 2008) and the epithelial homeostasis, too (Rakoff-Nahoum et al., 2004). For example, MyD88^{KO}mice showed significant differences in the composition of the distal gut microbiota (Wen et al., 2008) and had a significant lower Firmicutes/Bacteroidetes ratio compared to the control group. Since these impaired host innate immune responses can directly be linked to disturbances in composition of the microbiota, it has been hypothesized (Salzman et al., 2007) that effector molecules of the innate immune system, like antimicrobial peptides, are involved in regulating the composition of the microbiota.

In the absence of bacterial colonization, axenic vertebrates display many defects, e.g. in the ability to fight infections (Brandl et al., 2008; Rakoff-Nahoum et al., 2004; Shanmugam et al., 2005), in the development of the immune system (Mazmanian et al., 2005; Umesaki et al., 1999) and in the development of the digestive tract (Bates et al., 2006; Hooper et al., 2002). Thus, in vertebrates developmental processes are thought to be strongly affected by the need of maintaining a persistent specific microbiota. The constant recognition of the commensal flora by Toll-like-receptors in mice plays a protective role in intestinal homeostasis (Rakoff-Nahoum et al., 2004) and a single microbial molecule, polysaccharide A, is implicated in protection from intestinal inflammatory disease (Mazmanian et al., 2008). A well studied example of bacterial influences on host development in an invertebrate model organism is the

symbiosis between the squid *Euprymna scolopes* and the bacteria *Vibrio fischeri* in the host light organ. In response to the symbiosis both the host and symbiont undergo marked developmental changes (Visick and McFall-Ngai, 2000). *Vibrio fischeri* releases tracheal cytotoxin (TCT), which acts in synergy with lipopolysaccharide (LPS) to trigger several changes in the morphology of the host light organ (Koropatnick et al., 2004; McFall-Ngai, 2002).

The freshwater polyp *Hydra* - a new model in host-microbe interactions

In *Hydra,* Rahat & Dimentman already showed in 1982, that bacteria have profound effects on host tissue proliferation (Rahat and Dimentman, 1982). *Hydra* polyps were sterilized by a mixture of antibiotics, and cultured under axenic conditions. These polyps were unable to proliferate asexually by budding. This effect could be rescued by inoculating the culturing medium with bacteria from stock culture of *Hydra*. From this phenomenon the following questions arise at the beginning of my thesis: what kind of bacterial species are associated with the hydra epithelium and are there identifiable core microbiota associated with given *Hydra* species?

In the last years more and more genetic sequence data could be acquired from organisms at the base of animal evolution shedding new light on the ancestral gene repertoire (Putnam et al., 2007; Rast et al., 2006; Srivastava et al., 2008). Comparative data from different taxa revealed that Cnidarians share many genes with the Bilateria (Miller et al., 2005; Miller et al., 2007; Putnam et al., 2007; Technau et al., 2005). Recently, a first genome draft from *Hydra magnipapillata* with 6 fold coverage was assembled and is now online available (http://hydrazome.metazome.net). Additionally to a still growing set of at present 163.000 expressed sequence tags (ESTs), the development of very useful molecular techniques like stable transgenesis (Wittlieb et al., 2006) has been achieved. Especially transgenesis offers new insights into the interplay of host and associated microbes. These facts turn *Hydra* into a valuable model to address certain aspects that are central in understanding host-microbe interactions. These include the mechanisms protecting and maintaining epithelia homeostasis in health and disease and the evolutionary relationship between innate immunity and commensal microbes.

Phylogeny – at the base of animal evolution

Being the sister group of the Bilateria, Cnidaria represent a basal animal group (Collins, 2002; Martindale et al., 2002; Putnam et al., 2007) (Figure 1.1). Members of this taxon are characterised by a phylotypic cell type, the nematocyte or cnidocyte, one of the most complex cell types in the animal kingdom (Tardent, 1995).

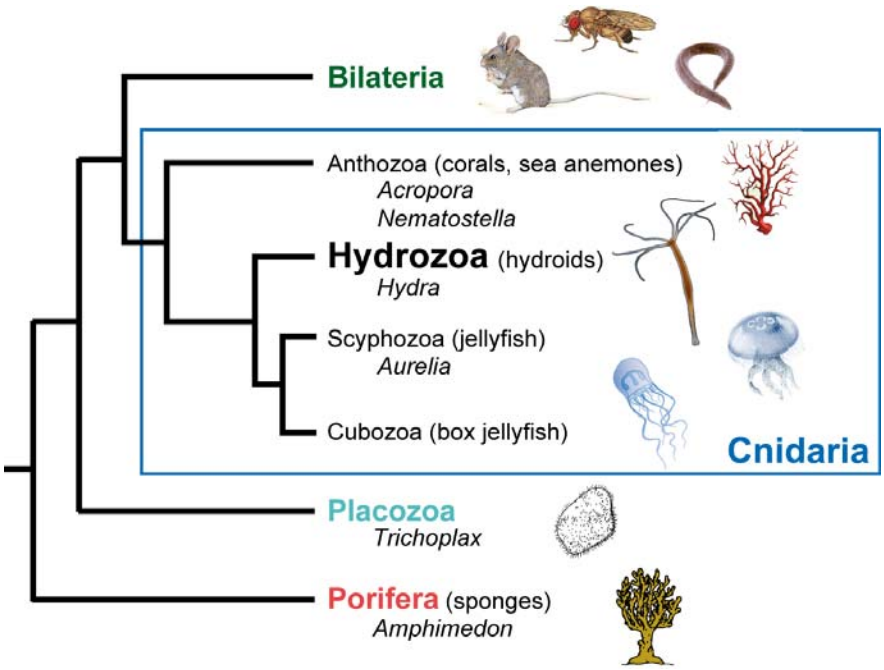

Figure 1.1. Schematic phylogeny of the 'lower' Metazoa. The Cnidaria are a sister group of all Bilateria. Anthozoa is the most basal class of Cnidaria.

The Cnidaria embody a diploblastic level of organisation. Their body is build up by two germ layers – endoderm and ectoderm. The mesoderm, a third germ layer typical for bilaterian animals, is not developed at this level of organisation. Cnidaria are the first phylum in the animal kingdom with true epithelia, characterised by tight junctions and a simple nervous system (Nielsen et al., 1996). Two forms of body organisation occure within the phylum – the sessile polyp and the pelagic medusa. The Cnidaria are subdivided into the Anthozoa, the Cubozoa, the Scyphozoa and the Hydrozoa (Figure 1.1). The Anthozoa are regarded as basal within the Cnidaria (Collins et al., 2006; Medina et al., 2001). The Hydrozoans are seen as the most derived group based on their outstanding cnidocyte type complexity, body plan diversity and molecular evidence (Collins et al., 2006; Steele, 2002). The exact systematic classification of *Hydra* spec. is: phylum: Cnidaria; class: Hydrozoa; subclass: Hydroidolina;

order: Anthomedusae; suborder: Capitata; family: Hydridae. Recently, Hemmrich et al. have resolved the relationship of several species of the genus *Hydra* using two nuclear and two mitochondrial markers as well as morphological criteria (Hemmrich et al., 2007). This analysis identified *Hydra viridissima* harbouring an algae symbiont as the most basal and *Hydra magnipapillata* and *Hydra vulgaris* as the most derived species (Figure 1.2).

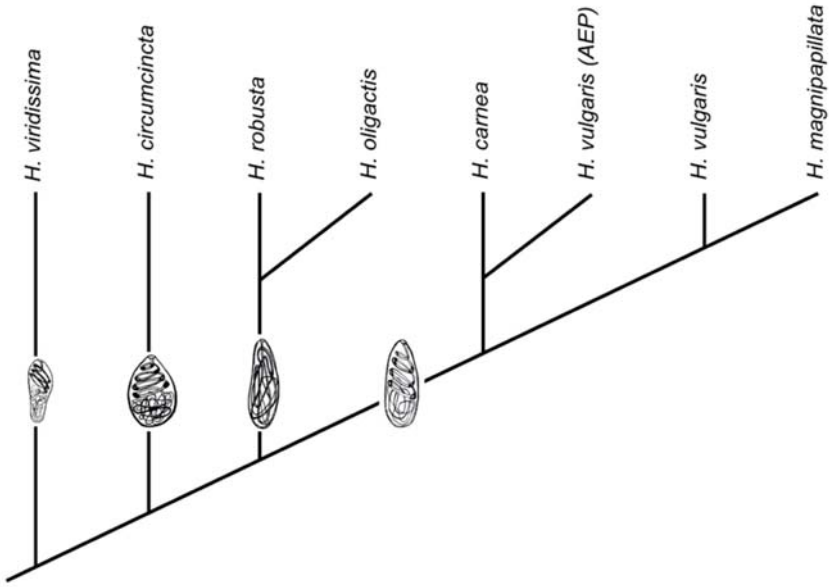

Figure 1.2. Phylogeny of *Hydra*. Phylogeny of different *Hydra* species as indicated by a phylogenetic analysis using two nuclear (18S, 28S rRNA) and two mitochondrial (16S rRNA, COI) genes. The holotrichous isorhizas of the different groups are depicted. Taken from (Hemmrich et al., 2007).

Biology – life in a freshwater pond

Hydra lives, unlike most hydrozoans, in freshwater ponds or lakes. The animals are attached to the substratum with their basal disk and feed on small crustaceans, like copepods or daphnia, which are captured by the means of cnidocytes located on the tentacles. Reproduction in *Hydra* takes place either asexual by budding (Clarkson and Wolpert, 1967), facilitating laboratory culture maintenance and propagation, or sexually by the formation of eggs and sperms (Bosch and David, 1987; Littlefield, 1985; Littlefield, 1991) (Figure 1.3). Sexual reproduction is regulated by environmental signals, but only in some species the signals are known, e. g. a decrease in temperature in *Hydra oligactis* or starvation in *Hydra vulgaris* (AEP). During sexual reproduction male polyps develop sperm-releasing testis (Figure 1.7A) and female polyps develop external eggs (Figure 1.7B). Being attached to the mother polyp, the

eggs get fertilized by swarming sperms (Martin et al., 1997). Some *Hydra* species are hermaphroditic and develop testis and eggs simultaneously, e. g. *Hydra circumcincta* and *Hydra viridis* (Holstein, 1995).

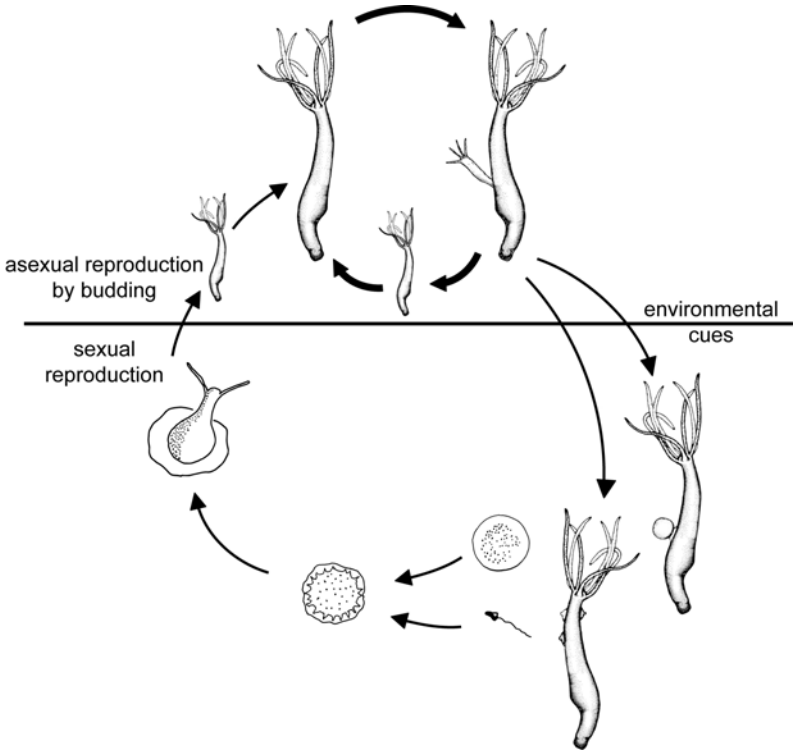

Figure 1.3. Lifecycle of *Hydra*. Asexual reproduction takes place by budding, sexual reproduction is regulated by different environmental cues.

Morphology and histology – a simplified body plan

The body of *Hydra* is simple, consisting of a tube with a food structure referred to as basal disk for adhesion to substrate and an apical structure comprising a hypostome surrounded by a ring of tentacles (Figure 1.4 A). The two epithelial cell layers in *Hydra*, the endoderm and the ectoderm (Figure 1.4 B), derive from separate lineages of epithelio-muscle stem cells. The epithelial cells in the adult *Hydra* are constantly proliferating. The cells differentiate, while changing their relative position along the body axis (Figure 1.4 A). Both epithelial layers are separated by an acellular layer, the mesoglea (Figure 1.4 B). A population of self-maintaining multipotent interstitial cells (i-cells) resides within the ectoderm throughout the gastric region giving rise to a variety of specialized somatic cells (nerve cells, nematocytes, gland cells) and germ cells (Figure 1.5) (Bode, 1996; Bosch, 2007). The different cell types of *Hydra* are

lineage restricted. Neither interconversion between cells from the interstitial lineage and the epithelial cell lineages, nor between the ectodermal and endodermal epithelial cells has been observed (Sugiyama and Fujisawa, 1978).

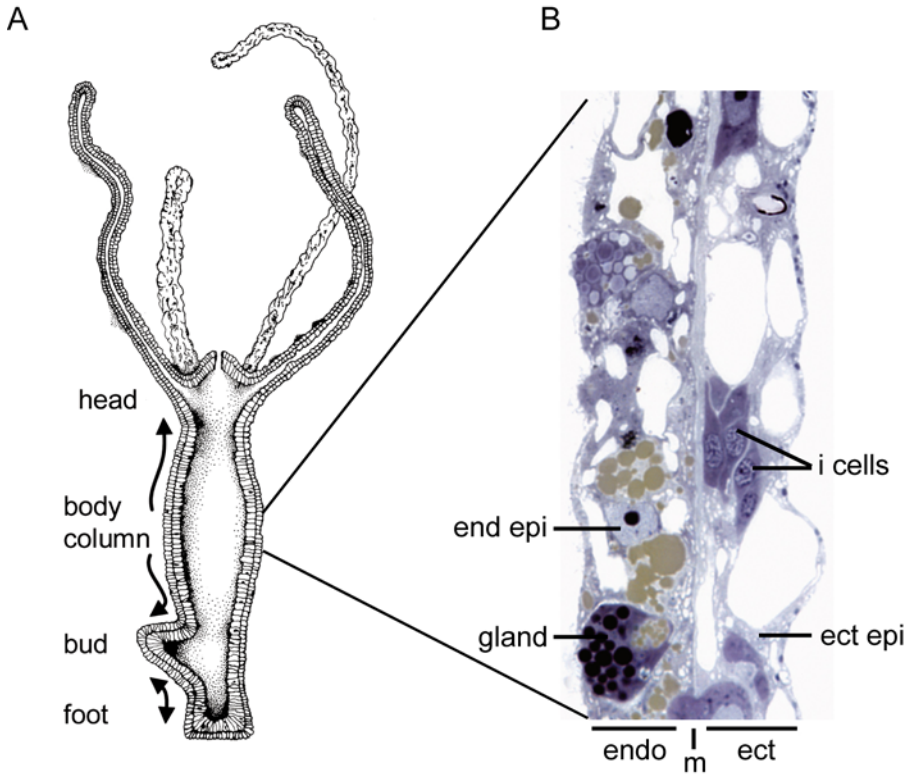

Figure 1.4. Morphology and histology of *Hydra*. (A) Schematic representation of a *Hydra* polyp. (B) Cellular composition of *Hydra* tissue within the gastric region. end epi: endodermal epithelial cell, gland: zymogen gland cell, ect epi: ectodermal epithelial cell, i-cells: interstitial cells, end: endoderm, ect: ectoderm, m: mesoglea. Modified from (Bosch, 2007).

The nervous system of *Hydra* is simple and has a netlike structure with high densities of neurons in the head and the foot (Bode et al., 1973). It consists of nerve cells with sensory or ganglionic character residing between epithelial cells of both layers.

The special effects of certain cell types in the epithelium on the associated microbial community are understood neither in vertebrates nor in invertebrates. Thus, the question arise which influences have specific changes in hydra tissue composition on the associated microbiota.

Gametogenesis and embryogenesis – without a separate germ line

In *Hydra* the germ line is not separated from the somatic cell lineage. The interstitial stem cells are located between the ectodermal epithelial cells throughout the gastric region and are showing stem cell properties. The main evidence for their stem cell properties comes from in vivo cloning experiments showing that these cells are multipotent and capable for somatic and germ line differentiation (Bosch, 2007; Bosch and David, 1987) (Figure 1.5).

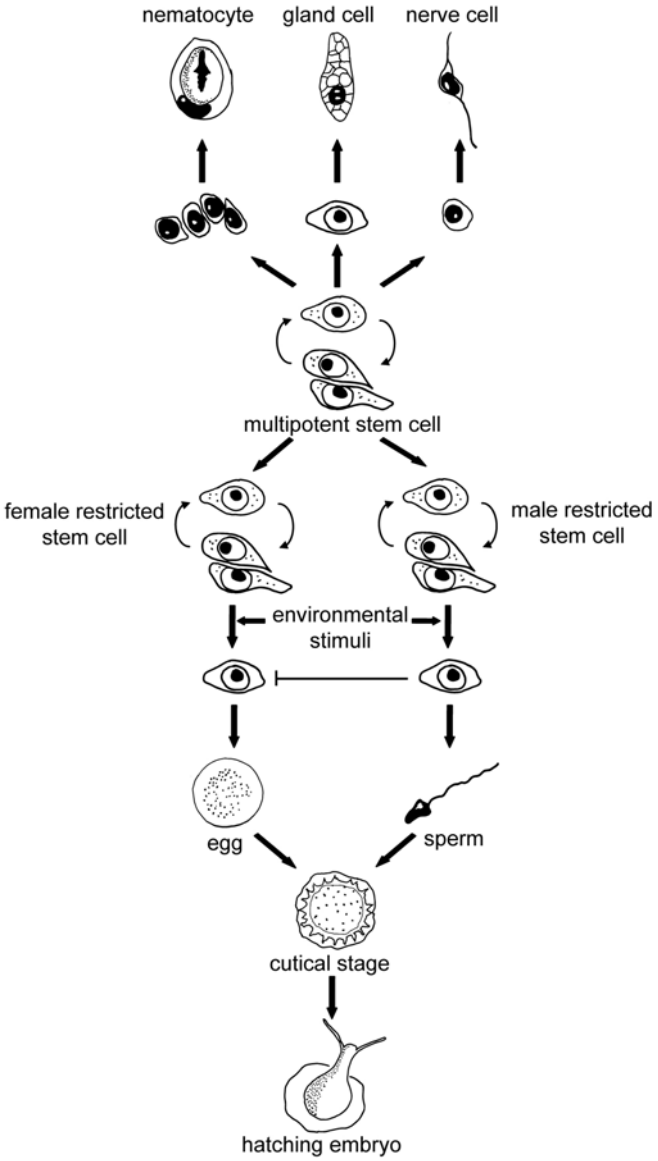

Figure 1.5. Model for differentiation of interstitial stem cells in *Hydra*. Multipotent stem cells give rise to somatic cells (nematocytes, gland cells and nerve cells) and germ line cells. The germ line differentiation is controlled by environmental stimuli. Modified from (Littlefield, 1991).

A unipotent subpopulation of interstitial cells are capable of proliferation but committed to either spermatogenesis (Littlefield, 1985; Nishimiya-Fujisawa and Sugiyama, 1993) or

oogenesis (Littlefield, 1991; Nishimiya-Fujisawa and Sugiyama, 1995). These germ line precursor cells are also present in low numbers in asexually proliferating polyps (Holstein and David, 1990) and undergo enhanced proliferation and differentiation into gametes in response to environmental stimuli (Figure 1.5). In addition, male germline cells suppress the ability of female germline cells to differentiate into eggs leading to "masculinisation" of females (Littlefield, 1994; Sugiyama and Sugimoto, 1985).

Spermatogenesis is initiated by local accumulations of interstitial cells within the intercellular spaces of ectodermal epithelial cells (Tardent, 1974). Clusters of accumulated interstitial cells synchronously undergo meiotic divisions followed by spermatogenesis (Munck and David, 1985) and the developing of external testis (Figure 1.7 A). While the differentiation pathway as well as the structure of the mature spermatozoa is very similar in *Hydra* and higher metazoa, nothing is known about the temporal and spatial signals controlling spermatogenesis.

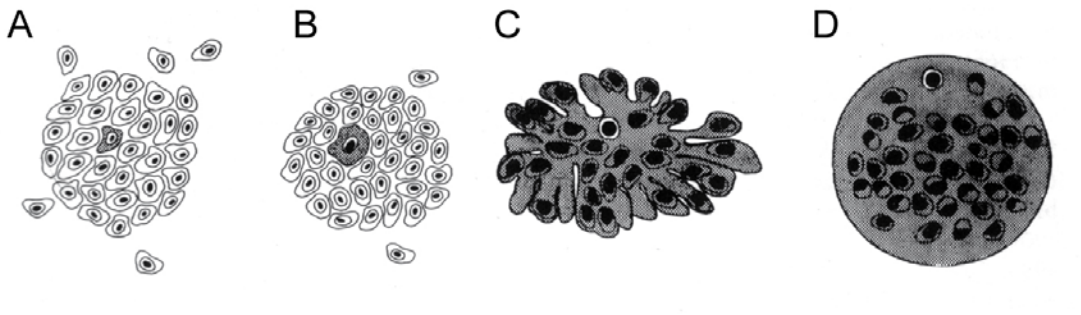

Figure 1.6. Scheme of *Hydra* oogenesis. (A) Accumulation of interstitial stem cells and determination of one oocyte. (B, C) Growth of oocyte by phagocytosis of nurse cells. (D) mature oocyte. Modified from (Holstein, 1995).

Oocytes differentiate from clusters of interstitial cells committed to the egg lineage. Within each cluster, one of the cells develops into an oocyte (Figure 1.6 A, B), while the other interstitial cells, often referred to as nurse cells are phagocytosed (Figure 1.6 C) and become incorporated into the cytoplasm of the developing oocyte (Honegger et al., 1989; Martin et al., 1997; Tardent, 1974). These condensed nurse cells constitute the bulk of the ooplasm, persist throughout embryogenesis (Figure 1.6 D) and provide most likely nutrients for the developing oocyte, as can be expected from the high content of glycogen particles and lipid droplets in their cytoplasm (Honegger et al., 1989).

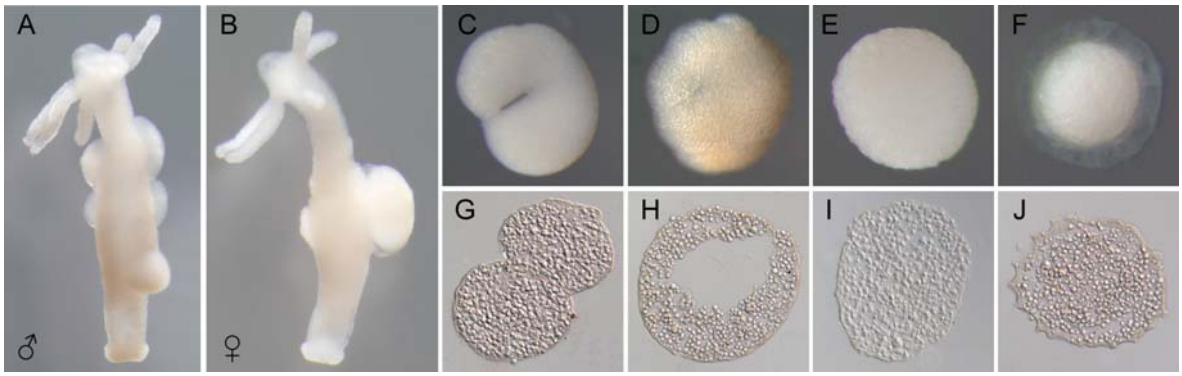

Figure 1.7. *Hydra* embryogenesis. (A) Male *Hydra* polyp with testis. (B) Female *Hydra* polyp with egg. (C, G) First cleavage. (D, H) Coeloblastula. (E, I) Gastrula. (F, J) Cuticle stage embryo,

Following fertilization, oocytes develop by a holoblastic, radial cleavage pattern (Figure 1.7 C, G) into a coeloblastula (Figure 1.7 D, H) (Martin et al., 1997). Embryos gastrulate by multipolar immigration (Figure 1.7 E, I) resulting in the definition of the two germ layers, ectoderm and endoderm. Unlike most other cnidarians, gastrulation in *Hydra* is followed by a cuticle stage (Figure 1.7 F, J). During this stage the embryo is surrounded by a thick protective outer layer, commonly referred to as embryotheca (Martin et al., 1997) and persists in diapause. Finally, a young polyp directly hatches from the cuticle stage, thereby omitting the typical planula larva stage (Martin et al., 1997).

Innate immunity – protection at the epithelial interface

To protect themselves against pathogens, vertebrates have developed a complex immune system that integrates fast innate defence mechanisms with delayed adaptive responses. Crucial elements of innate immunity in vertebrates include the Toll-like receptors (TLRs) (Leulier and Lemaitre, 2008) and the NOD-like receptors (Fritz et al., 2006), which detect conserved pathogen-associated molecular patterns (PAMPs), like LPS or flagellin, to initiate a complex antimicrobial response (Akira et al., 2006). Invertebrates rely exclusively on innate host defence systems to prevent infectious agents from entering the body (Beutler, 2004).

Despite its morphological simplicity and the lack of specialized phagocytes, *Hydra* has complex epithelial cell-based mechanisms for host defence. As shown in Figure 1.8, exposure of *Hydra magnipapillata* polyps to filtrates of adherent grown *Pseudomonas aeruginosa* causes striking changes in ectodermal epithelial morphology (Bosch et al., 2008).

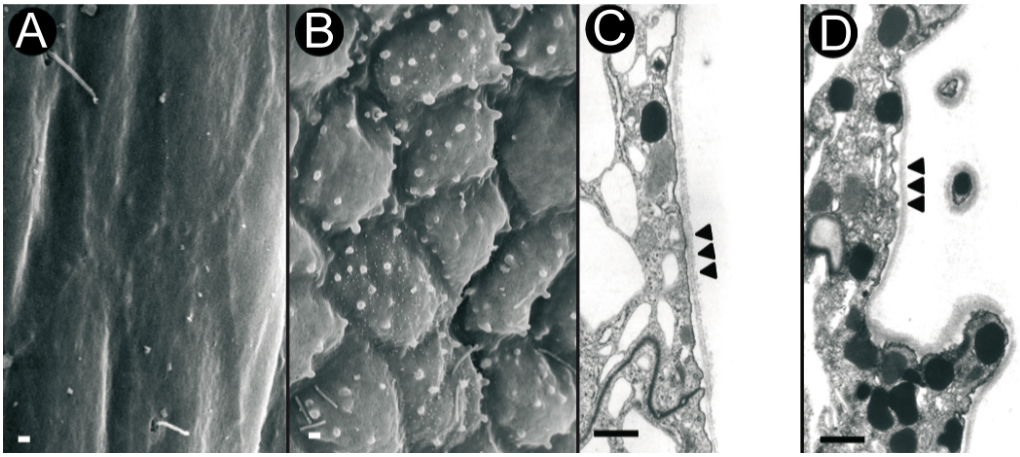

Figure 1.8. Epithelial response to pathogen exposure. (A) REM picture showing the ectodermal epithelium in control polyps; (B) REM showing the ectodermal epithelium in polyps exposed to filtrates of adherent grown *Pseudomonas aeruginosa* (*P..a.*); (C) TEM showing ectodermal epithelial cells in control polyps. Arrowheads indicate outer cell membrane and glycocalix. (D) TEM showing ectodermal epithelial cells in *P.a.* filtrate-challenged polyps. Note the large number of intracellular granules. Arrowheads indicate outer cell membrane and glycocalix. Modified from (Bosch et al., 2008).

Ectodermal epithelial cells from stimulated polyps round up and form numerous blebs at the cell surface (Figure 1.8 B). Analysis of thin sections indicates that epithelial cells in *P. a.* filtrate-exposed *Hydra* polyps (Figure 1.8 D) contain a large number of granules. Thus, similar to immune cells in higher organisms (Zasloff, 2002), hydra epithelial cells appear to respond to PAMPs by cytoskeletal rearrangement and increased secretory activity of antimicrobial peptides (AMPs) (Bosch et al., 2008)

Toll-like receptors - unconventional way of recognition

The first described member of the broad family of Toll-like receptors was Toll, a receptor essential for the development of embryonic dorsoventral polarity in *Drosophila* (Anderson et al., 1985). Analysing the homologous receptors in mammals revealed 12 members of the family without any function in developmental processes. Functional studies of Toll-like receptors (TLRs) in mammals revealed an essential role in direct recognition of microbial components (Akira et al., 2006). Later it could be shown that Toll of *Drosophila* also plays a crucial role in antifungal response (Lemaitre et al., 1996) in addition to its function in development.

TLRs are type I integral membrane glycoproteins characterized by an extracellular domain containing variable numbers of leucine-rich-repeat (LRR) motifs. In vertebrates, the

intracellular domain of TLR is linked to the MyD88 dependent signalling cascade leading to the nuclear translocation of nuclear factor- kappa B (NF-κB) (Akira et al., 2006). The transcription factor NF-κB can induce pro-inflammatory cytokines, like IL-6 and TNFα (Akira et al., 2006) and a broad range of antimicrobial peptides (Birchler et al., 2001; Takeda et al., 2003). But the microbial ligand recognized by TLRs are not only unique to pathogens, but are also produced by commensal and mutualistic microorganisms. It could be shown that the recognition of commensal microorganisms via TLR plays a crucial role in the maintenance of intestinal epithelium homeostasis in mice (Rakoff-Nahoum et al., 2004). This leads to the assumption, that TLRs are not only involved in pathogen recognition, but also play a crucial role in host-microbe homeostasis.

TLRs are evolutionarily conserved receptors found in both vertebrates and invertebrates like *Drosophila* and the cnidarian *Nematostella vectensis* (Akira et al., 2006; Leulier and Lemaitre, 2008; Miller et al., 2007; Sullivan et al., 2007). As described in Bosch and Augustin et al. (2008), in *Hydra* no *bona fide* Toll-like receptor could be identified. Only two genes containing a Toll/ interleukin-1 receptor (TIR) domain, a transmembrane domain, and an extracellular domain lacking any specific domain structure, termed as Toll-receptor-related 1 (hyTRR-1) and Toll-receptor-related 2 (hyTRR-2), could be isolated from the *Hydra magnipapillata* EST collection. Additionally two full length transcripts were identified that encode putative transmembrane proteins carrying TLR-related LRRs on the extracellular part, termed as hyLRR-1 and hyLRR-2. In human HEK293 cells, it could have been shown that the hyLRR2 recognizes the bacterial PAMP flagellin and that only the receptor complex between hyTRR-1 and hyLRR-2 is able to activate an immune response by an increase of NF-κB activation (Bosch et al., 2008).

Antimicrobial peptides – an evolutionary ancient invention

Antimicrobial peptides (AMPs) are the effector molecules of the innate immune system and play a crucial role in host defence reactions. All animals produce AMPs as part of the first line of defence (Wang and Wang, 2004). They share similar characteristic features; they are relatively short, positively charged and amphiphilic (Jenssen et al., 2006). Despite their similar general physical properties, individual peptides show limited sequence homologies and a wide range of secondary structures (Jenssen et al., 2006). Many eukaryotic AMPs are expressed as

prepropeptides and stored after proteolytic cleavage of the signalpeptide as an inactive propeptide. The mature active AMP is released after a second proteolytic cleavage (Shinnar et al., 2003) leading to a N- and C- terminal fragment. Cationic host defense peptides show the ability to kill or inhibit bacteria (Zasloff, 2002), inhibit viral infection (Albiol Matanic and Castilla, 2004; Wachinger et al., 1998), and lyse fungal cell wall (De Lucca and Walsh, 1999). The mode of action is well understood for peptides with antibacterial activity. Two major mechanisms are described in the literature: killing of bacteria (i) due to permeabilization of the membrane and (ii) due to inhibition of intracellular processes (Jenssen et al., 2006).

Until today two potent AMPs, hydramacin and periculin, have been identified in *Hydra* (Bosch et al., 2008). The first identified AMP in *Hydra* was hydramacin1 containing a putative signal sequence directly followed by the mature peptide with a basic cationic 60 aa peptide containing eight cysteines with a calculated monoisotopic molecular mass of 7009 Da. *Hydramacin1* mRNA is expressed exclusively in endodermal epithelium. Low concentrations of LPS upregulate *hydramacin1* expression, indicating, that hydramacin1 is inducible by microbial products (Bosch et al., 2008).

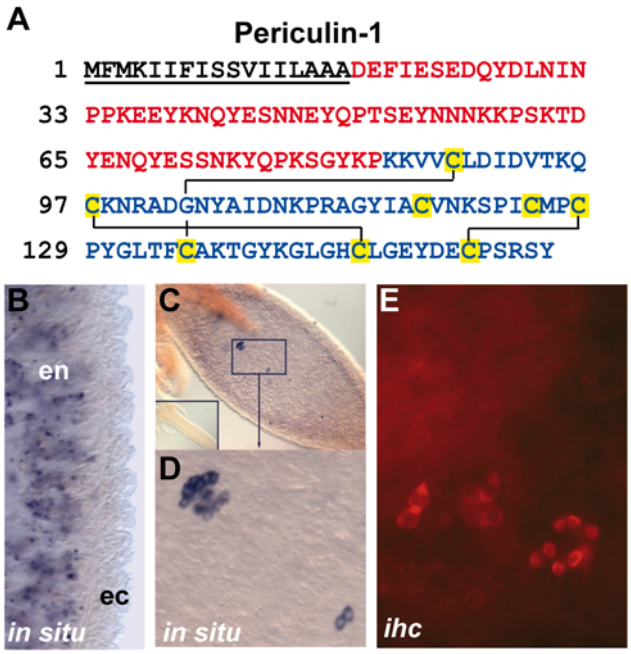

Figure 1.9. Periculin-1, a novel host defence effector molecule in *Hydra*. (A) Periculin-1 amino acid sequence and structural features. A signal peptide (underlined) is followed by an anionic (red amino acid residues; pI value 4.5) and a cationic (blue amino acid residues, pI value 8.7) domain which contains 8 cysteines (marked in yellow) predicting three disulfide bridges (solid lines); (B-D) *Periculin1* mRNA is expressed in endodermal cells (B) as well as in interstitial cells in the ectoderm (C-D); (E) polyclonal antiserum shows the periculin1 peptide localized in the endoderm as well as in some ectodermal interstitial cells. Modified from (Bosch et al., 2008).

The second AMP in *Hydra* was identified by using suppression subtraction hybridisation (SSH) of polyps induced with filtrates of adherent grown *Pseudomonas aeruginosa* substracted from control polyps (Bosch et al., 2008). Among the genes upregulated by *P. a.* filtrates, the gene *periculin1* was discovered, termed due to its rapid response to a wide variety of bacterial and tissue "danger" signals. Analysis of the deduced amino acid sequence of periculin1 and the charge distribution within the molecule reveals an anionic N-terminal region and an 8 cysteine residues containing cationic C-terminal region (Figure 1.9 A). *Periculin1* is expressed in the endodermal epithelium as well as in some interstitial cells in the ectoderm (Figure 1.9 B-D). Immunofluorescence staining identified periculin1 peptide in endodermal epithelial cells as well as in a subpopulation of ectodermal interstitial cells (Figure 1.9 E). To assess the antimicrobial activity of periculin1, recombinant peptide was generated representing the cationic C-terminal region (Figure 1.9 A) in *E. coli*. When used against *Bacillus megaterium* ATCC14581, a bactericidal activity (LD90) of 0.2 – 0.4 µM was measured indicating that this peptide is also involved in *Hydra* host defence (Bosch et al., 2008). *Periculin1* expression is induced by several different stimuli (Bosch et al., 2008). Exposure of *Hydra* polyps to 2.5 µg/ml flagellin upregulates *periculin1* expression in a time dependent manner. Unexpectedly, introduction of dsRNA of unrelated sequence into *Hydra* polyps by electroporation also caused a strong upregulation of *periculin1* expression (Bosch et al., 2008). Cell death plays an important role in *Hydra* tissue homeostasis (Bosch and David, 1984; Cikala et al., 1999) and may also be responsible for removal of infected cells. A high level of *periculin1* expression accompanies also induced cell death in *Hydra*. In vertebrates, one of the principal endogenous immunological "danger signals" released from injured or dying cells is monosodium urate (MSU), the end product of purine catabolism (Matzinger, 2002). To investigate whether MSU can trigger *periculin1* expression, *Hydra* polyps were exposed to MSU for 24 hours. Unexpectedly, expression of *periculin1* was strongly upregulated after stimulation with MSU (Bosch et al., 2008). Thus, uric acid appears to be a highly conserved endogenous danger signal. In summary, the results indicate that a wide variety of bacterial and tissue components affect the expression of *periculin1* (Bosch et al., 2008).

From these results Bosch et al. (2008) propose a model for pathogen recognition in *Hydra.* The direct recognition of PAMPs is mediated by atypical transmembrane receptors with the extracellular LRR and the intracellular TIR domain being present on two different proteins

(Figure 1.10) (Bosch et al., 2008). Thereafter, the recognition of flagellin and other PAMPs leads to the secretion of AMPs, like periculin1.

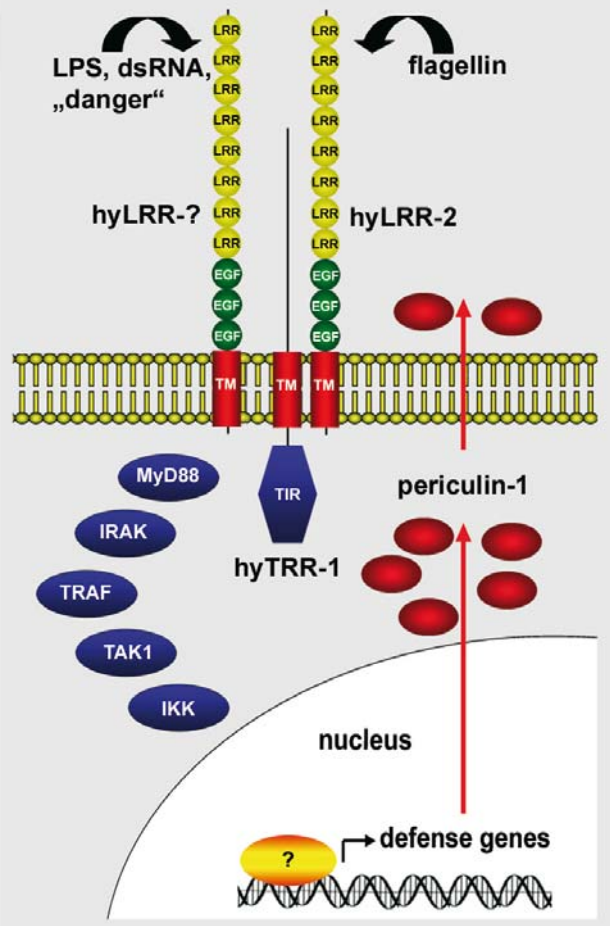

Figure 1.10. Molecular components of the pathways involved in the *Hydra* epithelial host defence system. Taken from (Bosch et al., 2008).

From the assumption that the innate immune system as the host's first line of contact with the microbiota plays a crucial role in shaping the microbiota, I ask the question if the identified AMPs from *Hydra* are involved in shaping the associated microbiota.

Aims of the study

Based on these facts and considerations, I tried to answer the following questions in the present thesis:

1. Are there identifiable core microbiota associated with given *Hydra* species?
2. Does disturbance of hydra tissue homeostasis affect the associated microbiota?
3. How are the microbiota selected, and how did they evolve within and between *Hydra* species?

Particular emphasis was on:

3.1. Which roles do innate immunity factors such as antimicrobial peptides play in *Hydra*-bacteria coevolution?

3.2. Which roles do environmental factors play in *Hydra*-bacteria association?

Chapter I: Long-term maintenance of species-specific bacterial microbiota in the basal metazoan *Hydra*

Introduction

Epithelia of all animals are colonized by complex communities of microbes (Steinhoff, 2005). Different epithelia within one organism have different microbial communities (Bik et al., 2006). Perturbations and imbalances in this usually beneficial relationship underlie many human diseases. In the absence of bacterial colonization, germ-free animals display defects in the ability to fight infections by pathogenic bacteria and viruses (Shanmugam et al., 2005). The mechanisms, which mediate the interdependent and complex interactions within microbial communities and the host epithelium, as well as the influence of the microbiota on immune functions, are not yet discovered. Molecular analysis of the bacterial microbiota in the human stomach (Bik et al., 2006) recently uncovered an unprecedented bacterial diversity which is different from the bacteria found in mouth or esophagus. In a reciprocal transplantation experiment with the microbial community of mice and zebrafish, evidence was provided (Rawls et al., 2006) that the gut epithelium is actively shaping the microbiota. To recognize and manage these complex communities of microbes, vertebrates, in contrast to invertebrates, are thought (McFall-Ngai, 2007) to have evolved adaptive immunity.

The microbiota living on or in invertebrate epithelia and their influence on the host immune system are largely unknown. Are there identifiable core microbiota associated with a given host species? How are the microbiota selected, and how did they evolve within and between hosts? Here I characterized bacterial diversity within the epithelia of the cnidarian *Hydra*. Cnidaria are one of the earliest branches in the animal tree of life, represent the simplest animals at the tissue grade of organization, and have a body plan in which there is no physical barrier between the host tissue and the microbes.

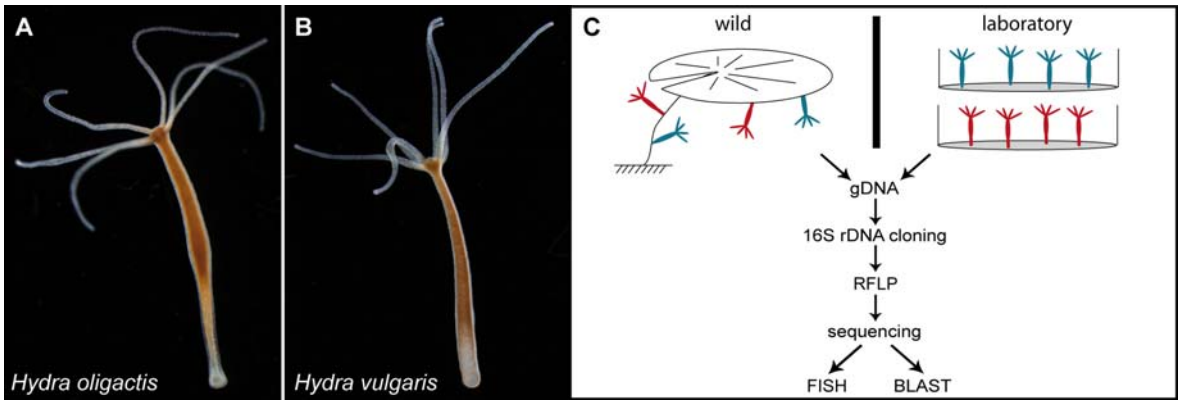

Figure 2.1. Analysis of *Hydra*-associated bacteria (A, B) Morphological characteristics of the two *Hydra* species analysed. (C) Schematic representation of the approach. Bacterial microbiota were compared between *H. vulgaris* (blue) and *H. oligactis* (red) from laboratory culture (right; drawn in plastic dishes) and the wild (left, attached to water lily). Modified from(Fraune and Bosch, 2007).

Thus, Cnidaria occupy an important evolutionary position for understanding direct host-microbe interactions. It was shown recently that the cnidarian immune system has evolved an elaborate mechanism to delete or suppress pathogenic intruders (Bosch et al., 2008; Miller et al., 2007). Research into molecules of the innate immune response in Cnidaria (Miller et al., 2007) have identified Pattern Recognition Receptors (PRRs), most notably the Toll-Like Receptors (TLRs) allowing permanent surveillance or resident microbiota and intruding pathogens (Bosch et al., 2008). Moreover, two closely related species of *Hydra*, *H. oligactis* (Figure 2.1 A) and *H. vulgaris* (Figure 2.1 B) are remarkably different in their stress response (Bosch et al., 1988; Brennecke et al., 1998). These differences appear to be correlated with differences in the antimicrobial activity against different bacteria (pers. observ.). Because this appears to be due to differences in selective constraints, I asked whether the species specific differences in the immune and stress response are reflected by the microbiota associated with these two *Hydra* species, and whether there is an identifiable specific core microbiota associated with a given species.

Results

The microbiota in two closely related *Hydra* species

To identify the microbiota in the *Hydra* epithelium, I performed a comprehensive bacterial DNA analysis in individuals of two *Hydra* species, *H. oligactis* (Figure 2.1 A) and *H. vulgaris* (Figure 2.1 B). Both species are closely related (Hemmrich et al., 2007)and located within the phylum Cnidaria. I compared 10 individuals from each species which were kept for more than 30 years under identical laboratory culture conditions with 10 *H. oligactis* individuals directly isolated from lake Pohlsee, 6 *H. oligactis* individuals isolated from lake Ploen and 6 *H. vulgaris* individuals isolated from lake Pohlsee. The experimental procedure is shown in Figure 2.1 C. For bacterial genotyping, 16S rRNA genes were amplified by PCR from each sample and cloned. From each sample, about 46 clones (see Table 1) were randomly selected for restriction fragment length polymorphism. A total of 68 16S rRNA sequences formed the final analyzed dataset.

Table 1. Bacterial species richness in *Hydra*

	N	S	Chao 1	
			mean ± SD	95% CI
Ho (lab)	77	3	4 ± 2	(3 – 16) (a)
Ho (lake Pohlsee)	34	4	5 ± 2	(4 – 17) (a, b)
Ho (lake Ploen)	54	5	5 ± 0	(5 – 5) (a)
Hv (lab)	44	12	17 ± 6	(13 - 41) (b, c)
Hv (lake Pohlsee)	39	12	19 ± 8	(13 - 54) (c)
Ho (medium)	43	25	43 ± 12	(30 - 85) (d)
Hv (medium)	46	22	35 ± 10	(26 - 70) (d)
lake Pohlsee (water)	43	23	30 ± 6	(25 - 51) (d)

Hv, *H. vulgaris*; Ho, *H. oligactis*; lab, animals taken from laboratory culture; lake Pohlsee, animals taken from lake Pohlsee; lake Ploen, animals taken from lake Ploen. N, total number of analysed clones; S, identified bacterial phylotypes; Chao1, richness estimator, significant differences are indicated by different letters. Modified from (Fraune and Bosch, 2007).

As shown in Figure 2.2, the restriction fragment length polymorphism patterns revealed drastic differences between the two *Hydra* species. In 75 out of 77 clones from *H. oligactis* from the long-term laboratory culture (Figure 2.2 A). I detected one dominant RFLP pattern, while in *H. vulgaris* individuals from the laboratory culture (Figure 2.2 C) 16 different RFLP patterns could be observed. The individuals from these cultures were exposed to constant

"environmental" conditions including culture medium, food and temperature for more than 30 years.

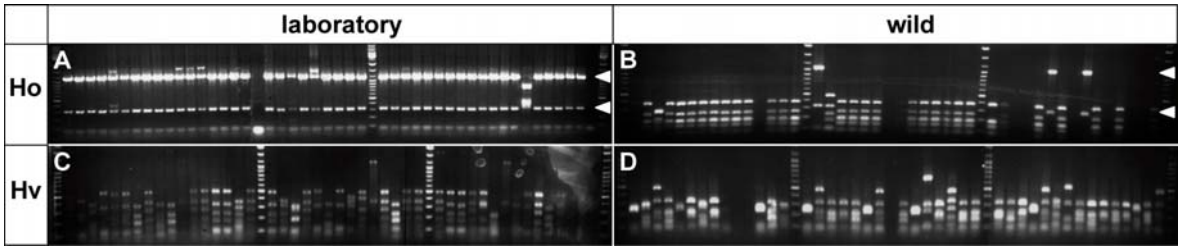

Figure 2.2. RFLP analysis of 16S rRNA genes of bacteria associated with two different *Hydra* species. (A) *H. oligactis* fom the laboratory culture, (B) *H. oligactis* isolated from the wild (lake Pohlsee), (C) *H. vulgaris* from the laboratory culture, and (D) *H. vulgaris* isolated from the wild (lake Pohlsee). Ho, *H. oligactis*; Hv *H. vulgaris*. Modified from (Fraune and Bosch, 2007).

Detecting drastic differences in the bacterial communities was, therefore, completely unexpected and indicated differences in selective constraints. To determine whether similar differences can be observed in *Hydra* individuals directly isolated from the wild, I performed bacterial genotyping in *H. oligactis* and *H. vulgaris* individuals isolated from two different lakes near Kiel. As also shown in Figure 2.2, the RFLP patterns from 6 individuals of *H. vulgaris* isolated from lake Pohlsee (Figure 2.2 D) shows similar complexity as the RFLP pattern in the laboratory animals (Figure 2.2 C). Moreover, and strikingly similar to the laboratory animals, in the *H. oligactis* polyps isolated from the lake (Figure 2.2 B) only one dominant RFLP pattern could be observed. Interestingly, although the bacterial RFLP pattern in the majority of *H. oligactis* polyps (72/89 clones analysed) from the wild seems to be different from the dominant RFLP pattern in the corresponding laboratory culture (see arrows in Figure 2.2 A, B), seven bacterial RFLP patterns from *H. oligactis* polyps from the wild show the identical RFLP pattern as the ones from *H. oligactis* polyps from the long-term laboratory cultures. Our results show not only that *H. vulgaris* polyps have a quite diverse microbial fauna while *H. oligactis* appear to be associated with only a limited number of microbes, but also that intra-species differences in the RFLP patterns of the samples from the wild and the long-term laboratory culture are much smaller than the differences between the two species.

Phylogenetic analysis reveals species-specific phylotypes

To identify the bacterial divisions in the *H. oligactis* and *H. vulgaris* epithelium, partial sequences (~1450 bp) were obtained from each RFLP type. After phylogenetic analyses were performed, sequences with ≥ 97% similarity were designated phylotypes. Figure 2.3 A shows the phylogenetic tree with the 36 phylotypes identified in the sequence data set. In the *H. vulgaris* samples from the lab and the wild, I identified in each case 12 different bacterial phylotypes. In *H. oligactis*, I identified 3 phylotypes in the laboratory culture and 4 respectively 5 phylotypes in the samples from the two lakes.

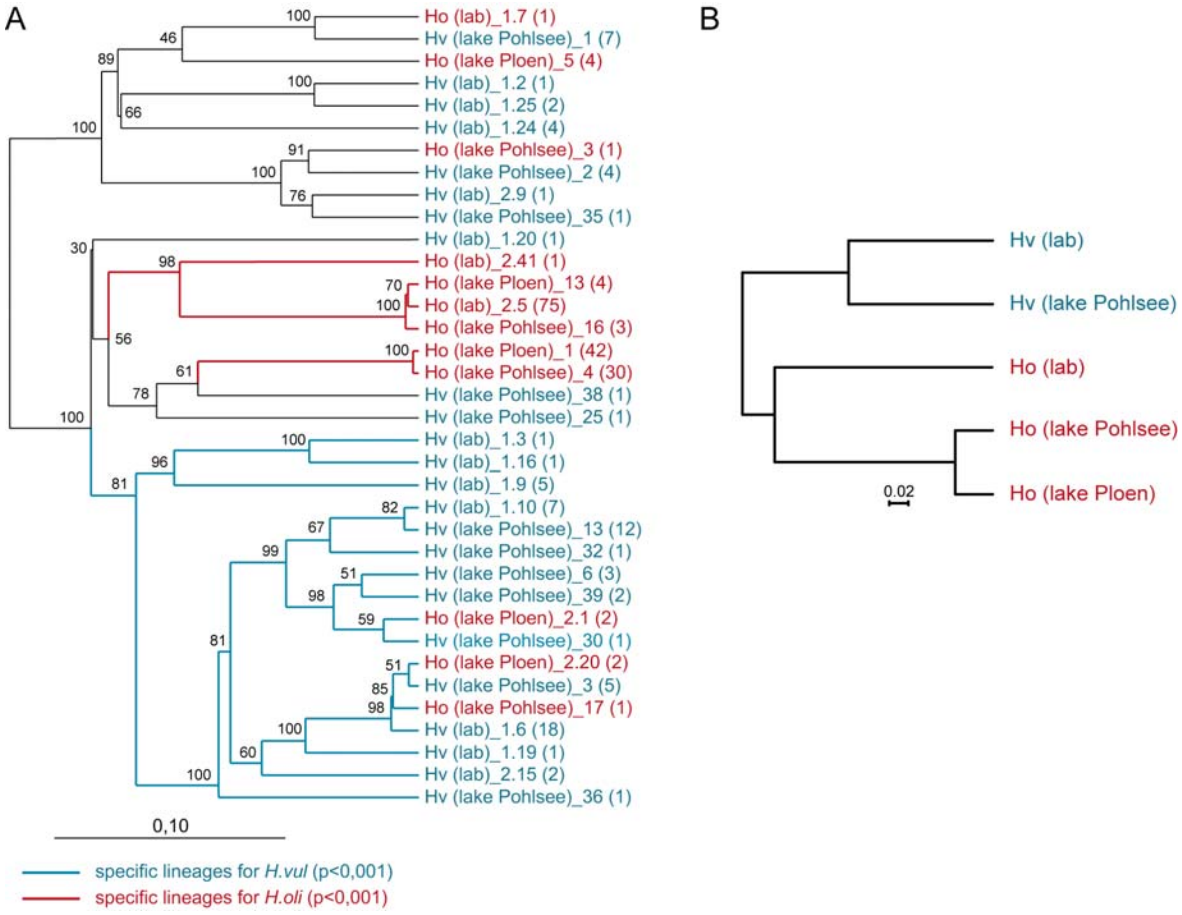

Figure 2.3. Phylogenetic comparison of identified bacterial phylotypes from the different *Hydra* species. (A) Neighbor-joining tree (Olsen correction) with the 36 identified 16S rDNA phylotypes from 5 different samples. Number of RFLP pattern within each phylotype are shown in parentheses. Bootstrap values are shown at the corresponding nodes (n = 100). The scale bar represents evolutionary distance (0.1 subsititutions per nucleotide). Specific bacterial lineages for the different *Hydra* species analysed with UniFrac are indicated in blue (specific for *H. vulgaris*, p<0.001) and red (specific for *H. oligactis*, p<0.001). (B) Jackknife environment cluster tree (weighted UniFrac metric, based on the 36 sequence tree; Lozupone et al., 2005) of the analysed bacterial communities. 100 jackknife replicates were calculated, each node was recovered with >99.9%. The scale bar shows the distance between the environments in UniFrac units. Hv, *H. vulgaris*; Ho *H. oligactis*; lab, animals from laboratory culture; lake Pohlsee, animals taken from lake Pohlsee; lake Ploen; animals taken from lake Ploen. Modified from (Fraune and Bosch, 2007).

To determine whether the observed microbiota of the two *Hydra* species are species specific, I processed the data by using two independent computational approaches. First, the bacterial communities of *H. vulgaris* and *H. oligactis* were compared by using the UniFrac computational tool (Lozupone and Knight, 2005). The UniFrac significance (using 1000 iterations; p=0.04) revealed a significant difference between both species. Additionally, the UniFrac Lineage-Specific Analysis, in which the relative abundance of each phylotype (Figure 2.3 A, in parentheses) is considered, identified specific bacterial divisions for the two different *Hydra* species (p<0.001). This indicates that the composition of the bacterial guilds is different in the two different species. Next, I tested the validity of these conclusions by performing UniFrac cluster analysis, which does hierarchical clustering analysis (also known as UPGMA) on the environments based on a distance matrix that is generated by calculating pairwise UniFrac values. As shown in Figure 2.3 B, the *H. vulgaris* datasets from both the laboratory culture and the wild clearly segregate from the datasets obtained from *H. oligactis*. These facts attest the resilience of the bacterial communities associated with the different *Hydra* species and indicate drastic compositional differences.

In the second approach I used EstimateS (Version 8, http://purl.oclc.org/estimates) to get insight into the bacterial species richness. As shown in Table 1, estimation of the number of phylotypes associated with both *Hydra* species by the nonparametric Chao1 algorithm (Chao, 1984) also revealed significant differences in the microbiota associated with both species. In the *H. vulgaris* samples from the laboratory and the wild I could identify 12 different phylotypes in each case. Therefore, Chao1 estimates a mean phylotype number of 17 and 19 for the *H. vulgaris* samples from the laboratory and the wild respectively. The number of identified phylotypes associated with *H. oligactis* was in all three samples significantly smaller than the one in *H. vulgaris*. As shown in Table 1, in *H. oligactis* from the laboratory I could identify three different phylotypes while in the *H. oligactis* samples taken from the lakes 4 (lake Pohlsee) and 5 (lake Ploen) phylotypes were detected. Thus, the species richness of bacteria appears to be lower in *H. oligactis* than in *H. vulgaris*. Moreover, phylotypes (RFLP patterns) associated with *Hydra* tissue could not be detected in the surrounding water.

Identification of multiple bacteria species within the hydra epithelium

To characterize the bacterial community, I analyzed the microbiota based on nearest relative (as determined by BLASTN search) and phylogenetic affiliation. As shown in Figure 2.4 and Figure 2.5, the 36 identified phylotypes represent three different bacterial divisions and are dominated by Proteobacteria and Bacteroidetes.

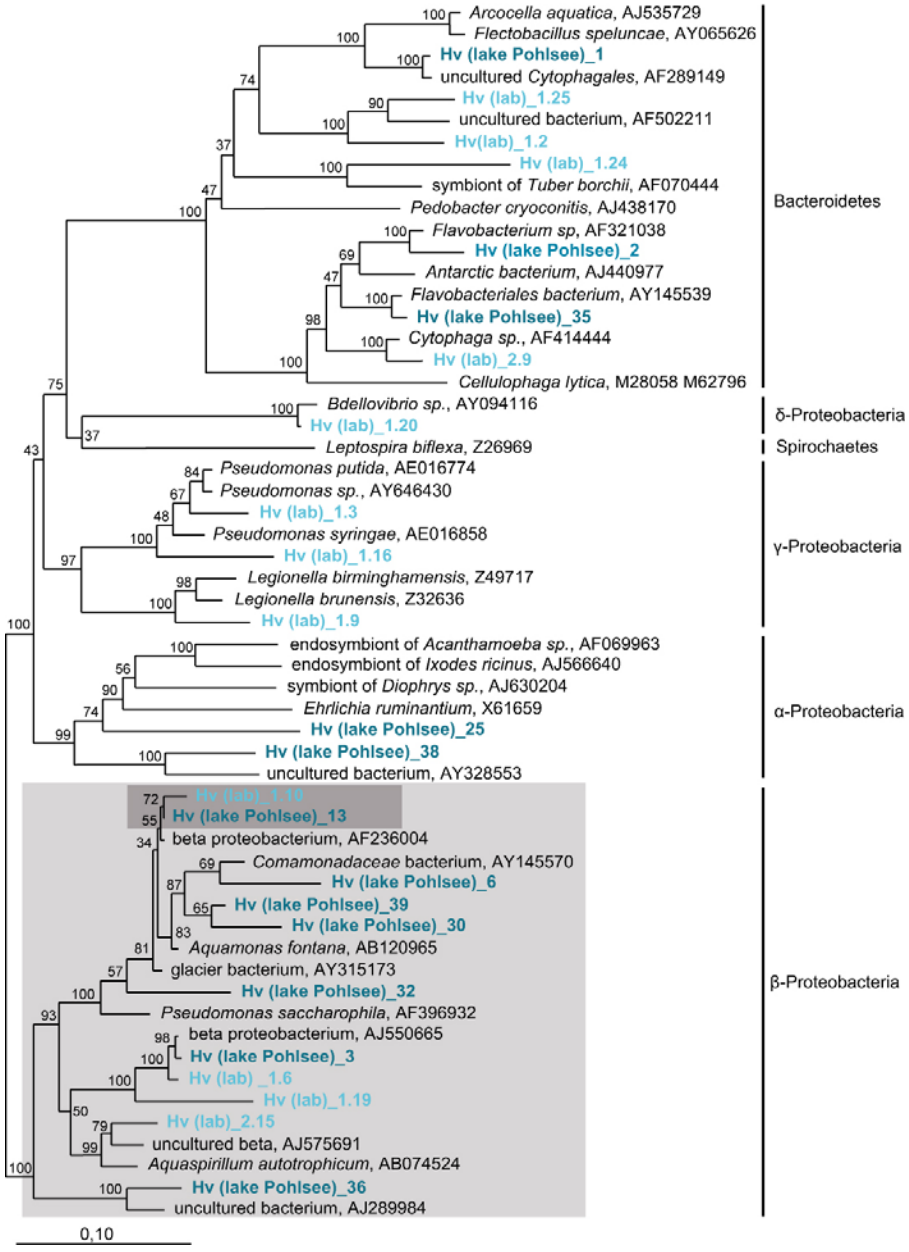

Figure 2.4. Phylogenetic position (16S rRNA gene sequences, neighbour-joining tree) of identified phylotypes associated with *H. vulgaris*. Light grey shadowed bacterial groups indicate species-specific bacterial guilds; dark grey shadowed bacterial phylotypes indicate species-specific bacterial species. The branch length indicator displays 0.1 substitutions per site. Modified from (Fraune and Bosch, 2007).

In *H. vulgaris* (Figure 2.4), the α-Proteobacteria are completely absent in the epithelium from laboratory cultured polyps, while γ-Proteobacteria (Pseudomonadales) are conspicuously absent in the samples from the wild. The Bacteroidetes and β-Proteobacteria are represented with several phylotypes in *H. vulgaris* tissue collected from both the laboratory and the wild. Interestingly, within the β-Proteobacteria, one phylotype [Hv(lab)_1.10 / Hv(lake Pohlsee)_13] (Figure 2.4, grey shadowed) appears to be species-specifically associated with *H. vulgaris*.

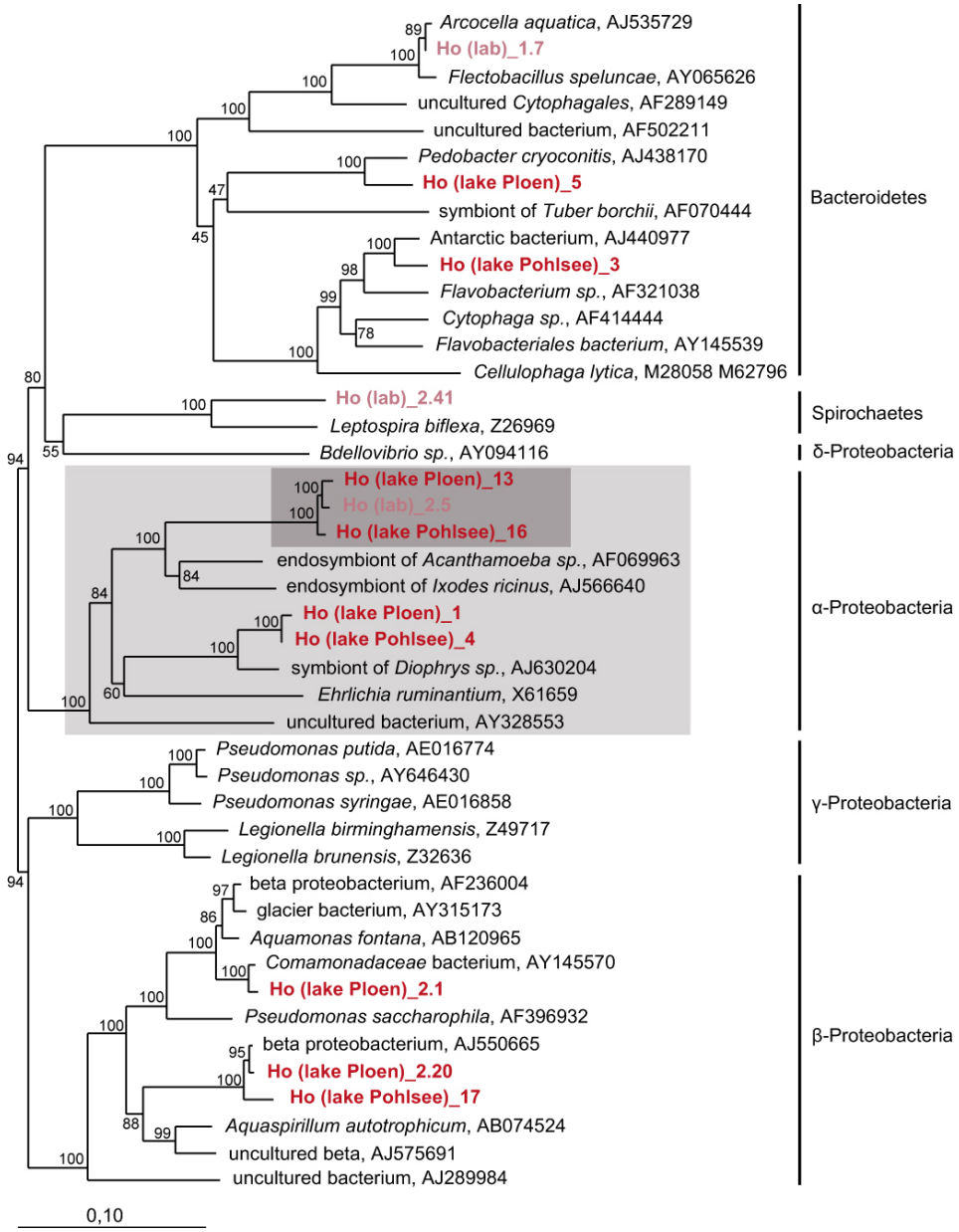

Figure 2.5. Phylogenetic position (16S rRNA gene sequences, neighbour-joining tree) of identified phylotypes associated with *H. oligactis*. Light grey shadowed bacterial groups indicate species-specific bacterial guilds; dark grey shadowed bacterial phylotypes indicate species-specific bacterial species. The branch length indicator displays 0.1 substitutions per site. Modified from (Fraune and Bosch, 2007).

Strikingly, this *H. vulgaris* specific bacterial phylotype was detected not only in the polyps from the long-term laboratory culture but also in *H. vulgaris* polyps directly isolated from the wild. This indicates that *H. vulgaris* actively maintains the association with this bacterial species. Moreover, and in sharp contrast to the data set obtained from *H. oligactis* (Figure 2.5), members of the β-Proteobacteria appear to be abundant in *H. vulgaris* tissue. In the bacterial phylotypes associated with *H. oligactis* (Figure 2.5), the majority of phylotypes belongs to the α-Proteobacteria (Rickettsiales). The molecular analysis shown in Figure 2.5 also indicates that within the α-Proteobacteria one phylotype appears to be species-specific for *H. oligactis*. This bacterial species was identified both in the sample from the laboratory culture and in polyps isolated from the wild. In *H. oligactis*, no phylotypes could be identified belonging to the γ-Proteobacteria. Taken together, these data show that both *Hydra* species select particular bacterial guilds. *H. oligactis* and *H. vulgaris* maintain these species-specific bacterial communities even when cultured under constant conditions for more than 30 years. Alternatively, it seems possible that the bacteria are also actively involved in selecting the host.

Discovery of endosymbiotic bacteria in epithelial cells of *H. oligactis* which are absent in *H. vulgaris*

Unexpectedly, the sequence analyses shown in Figure 2.5 indicated that the *H. oligactis* specific bacterial phylotype was most closely related to endosymbiotic bacteria. Since it was not known so far whether *H. oligactis* polyps contain endosymbionts and, if so, in which cells they were located, I analyzed the epithelial cells for the presence of endosymbiotic bacteria. The microscopic analysis (Figure 2.6 A, B) revealed numerous bacteria within all epithelial cells in all *H oligactis* polyps analyzed irrespective whether the animals were taken from the long-term laboratory culture (n = 15) or directly from the wild (n= 5). In contrast, no bacteria could be detected within the epithelial cells of *H. vulgaris* (data not shown). Transmission electron microscopy (Figure 2.6 C-D) revealed rod shaped bacteria of 2 to 4.5 µm size within the cytoplasm of *H. oligactis* ectodermal epithelial cells. As shown in Figure 2.6 D, the endosymbiotic bacteria are surrounded by a secondary membrane and are similar in morphology to bacteria identified previously as symbiotic bacteria in *Acanthamoeba* (Fritsche et al., 1999) or *Ixodes* (Beninati et al., 2004).

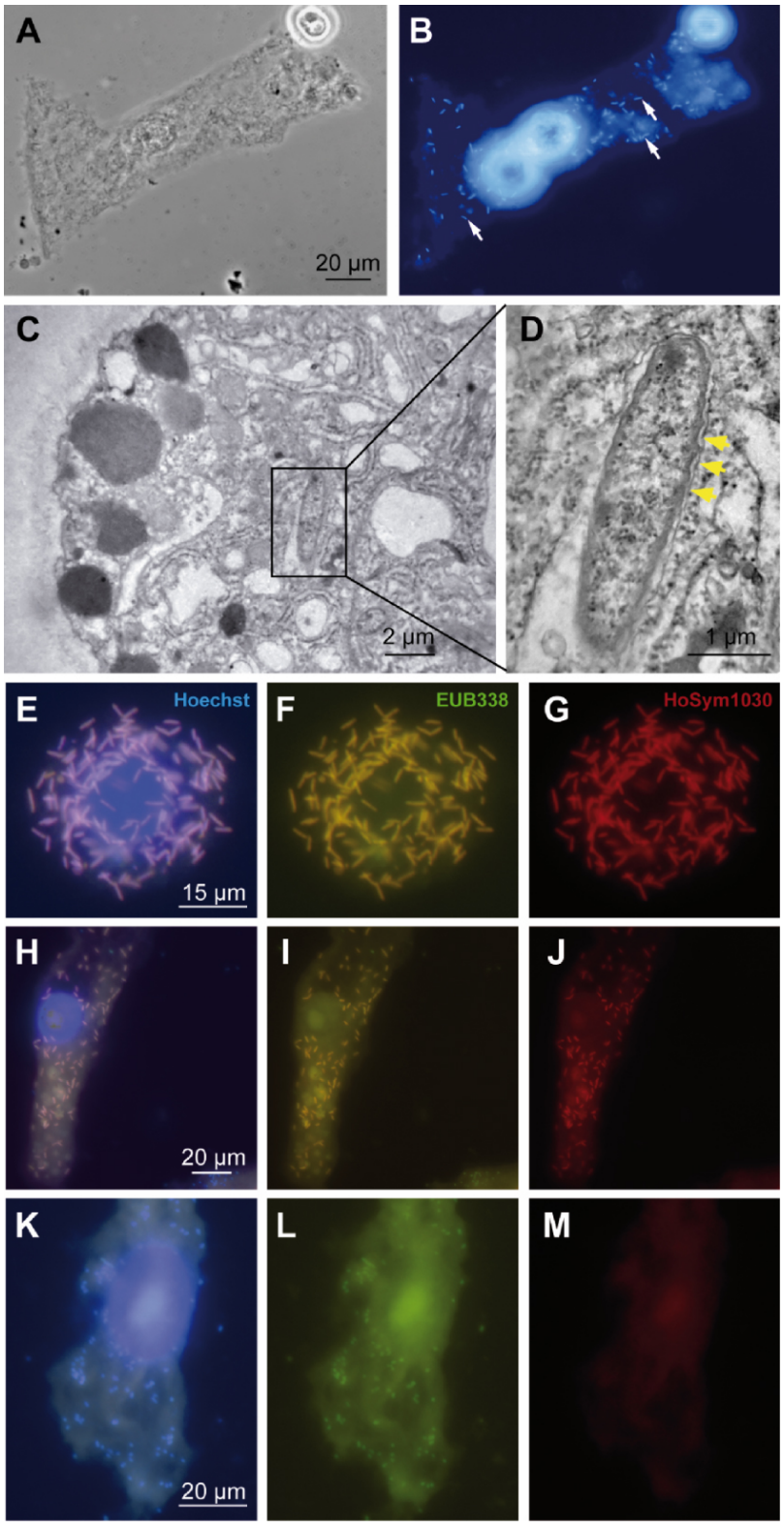

Figure 2.6. Microscopic analysis of endosymbiotic bacteria in *H. oligactis*. Macerated epithelial cell stained with Hoechst and evaluated with phase-contrast microscopy (A) and epifluorescence microscopy (B). (C, D) Transmission electron micrographs of bacterial endosymbionts in the cytoplasm of ectodermal epithelial cell. Secondary membrane is indicated by yellow arrow heads. (E-M) In situ hybridization (FISH) reveals endosymbiont identity. E-G show trypsin digested epithelial cells, H-M show macerated epithelial cells. In E, H and K cells were stained with Hoechst. In F, I and L bacteria cells were stained with the fluorescently labeled oligonucleotide probe EUB338 and in G, J and M the phylotype-specific probe HoSym1030. All cells were viewed with epifluorescence microscopy by using appropriate filter sets. Modified from (Fraune and Bosch, 2007).

To define the identity of the bacterial endosymbiont in *H. oligactis*, epithelial cells from animals cultured under laboratory conditions were subjected to whole-cell hybridization with fluorescence-labeled oligonucleotide probes (FISH). As shown in Figure 2.6, all Hoechst-stained bacteria (Figure 2.6 E) hybridised with a Eubacteria specific probe (EUB338) (Figure 2.6 F). Figure 2.6 G indicates that all these bacteria hybridize also with a probe (HoSym1030), which is specific for the *H. oligactis* specific bacterial phylotype described above (see Figure 2.5).

Next, I examined whether this putative symbiont was also present in epithelial cells of *H. oligactis* from the wild. Most strikingly - and shown in Figure 2.6 J, bacteria in ectodermal epithelial cells of *H. oligactis* isolated directly from the wild also hybridized to probe HoSym1030 indicating that the bacterial symbionts in polyps from the laboratory and the wild are closely related. However, in contrast to *H. oligactis* polyps from the long-term laboratory culture (Figure 2.6 E – G), in polyps isolated from the wild only a subpopulation of epithelial cells (about 20%) were found to harbour the putative symbiont detected by the HoSym1030 probe (Figure 2.6 H – J). As also indicated in Figure 2.6 L, all epithelial cells in *H. oligactis* polyps from the wild did contain endosymbiotic bacteria since they were detected by the eubacterial specific probe EUB338. Thus, both *H. oligactis* from the long term laboratory culture and from the wild do contain endosymbiotic bacteria. Polyps from the wild appear to contain at least two different endosymbiotic bacteria species (one of them is identical to the symbiont in the laboratory culture) whereas epithelial cells from the laboratory culture appear to contain a homogenous population of a single endosymbiotic bacteria species.

Transition from lake to laboratory causes a shift in the bacterial community in *H. oligactis*

To determine the impact of different environmental conditions on the bacterial community in *H. oligactis*, I cultured some of the polyps which were taken from the wild for 2 months under standard laboratory conditions and thereafter compared the bacteria-specific 16S rDNA RFLP pattern. As shown in Figure 2.7, culturing of *H. oligactis* from the wild under laboratory conditions has drastic effects on the composition of the bacterial community. While species of the α-Protobacteria which are the dominant species in the long term culture (indicated by arrow heads in Figure 2.7), can also be found in polyps 2 month and 6 month (data not

shown) after the shift to the laboratory, other bacteria disappear from the tissue collected in the wild.

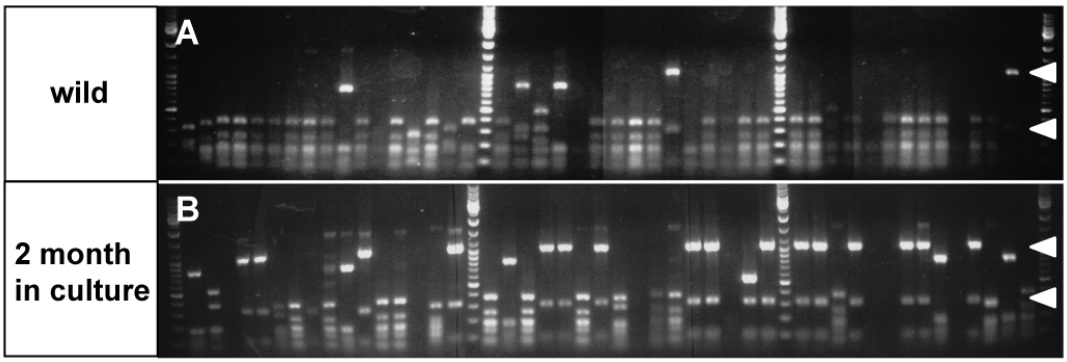

Figure 2.7. Comparative RFLP analysis of bacterial 16S rRNA genes associated with *H. oligactis* isolated from lake Ploen (A) and after cultivating in laboratory for 2 months (B). Taken from (Fraune and Bosch, 2007).

Figure 2.7 B also indicates the presence of microbes in the samples from the short-term cultures which were not detected in the samples from the wild. Thus, *H. oligactis* not only is associated with species specific symbiotic eubacteria but also responds to changes in the environment with changes in the bacterial community. Even under long-term and constant culture conditions, however, this bacterial community is very different from the community detected in *H. vulgaris* (see also Figure 2.2 B). Thus, *Hydra* appears to select their specific bacterial microbiota

Discussion

Because all epithelia in all animals including man appear to be colonized by microbial communities, it becomes important to understand the general principles by which these microbial communities evolve. Here, I describe the bacterial microbiota in epithelia from animals belonging to one of the earliest branches in the animal tree of life. Importantly, I show that in these animals the microbial communities are species specific. The unexpected observation that microbial communities were similar in *Hydra* polyps taken from the wild and polyps cultured for more than 30 years in the lab points to co-adaptive radiation of *Hydra* and its microbial communities.

In the absence of migratory phagocytic cells and any physical barrier between the epithelium and the microbes, the epithelium of the freshwater polyp *Hydra* is remarkably well equipped to survive in an environment teeming with potential pathogens (Bosch et al., 2008; Miller et al., 2007). The current study used molecular tools to reveal the previously uncharacterized bacterial microbiota in two species of *Hydra*. The *Hydra* epithelium was found to be colonized by a complex and dynamic community of microbes, and individuals from both species differed greatly in their microfauna. I also found that the bacterial species richness is significantly larger in *H. vulgaris* than in *H. oligactis*. Although my data show that the composition of the specific microbial communities associated with *H. oligactis* may vary in time (Figure 2.7), I observed an unexpected degree of similarity between the bacterial composition in individuals of laboratory cultures and polyps directly isolated from the wild (Figure 2.3 B). This indicates that the identified bacteria reflect resident species rather than transient "tourists" passing through with food, water and other environmental components. The differences in the microbial communities between the two species and the maintenance of specific microbial communities over long periods of time strongly indicate distinct selective pressures imposed on and within the *Hydra* epithelium, and suggest that the epithelium actively selects and shapes its microbial community.

Although this pilot study is far from being quantitative, it indicates that the number of bacterial species permanently associated with epithelia in *Hydra* is much less than, e.g., the number of resident bacterial species found in the human stomach or mouth (Bik et al., 2006).

This reinforces the idea (McFall-Ngai, 2007) that the diversity of resident bacterial communities is more complex in vertebrates than in invertebrates; and that this difference may have prompted the evolution of a memory-based adaptive immune system in vertebrates.

Since in *Hydra* (pers. observ.) as in vertebrates (Schreiber et al., 2005), differences in microbiota are correlated with differences in immune functions, it appears that epithelia may have an evolutionary requirement for the specific immunomodulatory direction provided by the microbiota. The observation (Rahat and Dimentman, 1982) that in the absence of bacteria *Hydra* polyps show strong developmental defects and are unable to proliferate asexually by budding, and that normal budding is resumed with non-sterile food or medium reinoculated with bacteria, supports the view that bacterial microbiota play a pivotal role in *Hydra*. It now will be important to assess the impact of the different microbiota on the innate immune system and to identify the environmental factors operating to select a specific bacterial community.

Depletion of microbiota appears to have profound effects in health and well being in both, hydra and humans. Thus, in both, hydra and humans, the immune system is likely to be strongly affected by the need to maintain a substantial resident microbiota. Genetic defects in the human immune systems ability to adequately respond to the epithelial microbiota appear to predispose individuals to inflammatory diseases (Schreiber et al., 2005). The results shown above provide compelling evidence for a complex cross-talk of an epithelial barrier and the residing microbiota at a basal level of evolution. In *Hydra*, this is complemented by unique biological and experimental opportunities. Thus, studies in *Hydra* may provide a paradigm for the characterization and analysis of microbial communities and their important, as-yet undiscovered roles in health and disease, and may reveal fundamental principles that underlie all host-microbe interactions.

Materials and Methods

Animal culture and collection

Experiments were carried out with *H. vulgaris* and *H. oligactis*. *H. vulgaris* was originally derived from the laboratory of A. Gierer (MPI Tübingen) while *H. oligactis* originally was obtained from P. Tardent (University of Zürich). The laboratory animals were cultured at standard conditions at 18°C for more than thirty years. Free living animals were isolated from lake Pohlsee (Schleswig-Holstein, Germany) and lake Ploen (Schleswig-Holstein, Germany) and cultured for 2 days in filtered water (0,2 µm) from the respective lake, to eliminate digestive food rests before DNA extraction. Species were identified by standard procedure (Holstein, 1995).

Molecular analysis

The whole animals were subjected to genomic DNA extraction using the DNeasy Tissue Kit (Qiagen, Hilden, Germany). Whole animals were lysed in 180 µl of DNeasy ATL buffer and 20 µl of proteinase K for 3 h at 56°C. 200 µl od AL buffer were added to the lysate and incubated for 10 min at 70 °C. After the addition of 200 µl 100% ethanol the lysates were purified over a DNeasy column and eluated in 100 µl of AE buffer. From the gDNA bacterial 16S RNA genes were amplified by PCR with the primers 27F (5'-TG(A/G)GTTTGATC(A/C)TGGCT(C/T)AG-3') and 1492R (5'-TGG(A/C/T)TACCTTGTTACGACTT-3') (Weisburg et al., 1991). PCR was conducted with 2.5 U Taq-DNA Polymerase (GE Healthcare) and its supplemented buffer system, 1mM of each primer, 0.1 mM of each dNTP and 1 µl of extracted DNA in a final volume od 50µl under a temperature profile of 94°C for 3 min followed by 30 cycles of 94°C for 30s, 53°C for 30s, and 72°C for 1 min 40s. Resulting PCR fragments were cloned into pGEMT vector (Promega, Madison, Wisconsin) and transformed into electrocompetent DH5α Escherichia coli cells (Invitrogen, Karlsruhe, Germany). The plasmid inserts were checked by PCR with the vector specific primers SP6 (5' ATT TAG GTG ACA CTA TAG AAT AC 3') and T7 (5' TAA TAC GAC TCA CTA TAG GG 3') for the correct product size (approximately 1600bp). The amplified inserts of the 16S rRNA genes were subjected to restriction fragment length polymorphism by using the restriction enzymes HaeIII and Hin6I (Fermentas).

Representative plasmids were sequenced using a LI-COR 4300 DNA Analyzer plate sequencer (LICOR Biosciences, Lincoln, Nebraska).

Phylogenetic data analysis

Sequences were sorted into phylotypes using the criterion of 97% sequence identity. Phylogenetic analysis revealed 36 phylotypes defined by 97% pairwise sequence identity. All the sequences were subjected to the Check chimera program Bellerophon (Huber et al., 2004) and RDP II Chimera Check (Cole et al., 2003) for the elimination of chimeric sequences. Chloroplast sequences were identified and removed. The final dataset of 36 sequences were aligned using the ARB software package (Ludwig et al., 2004). Closely related sequences were found by the function 'search for the closest relatives' implemented in the ARB software and by a BLAST search and added also to the alignment. Alignments were optimized by hand and a neighbor-joining tree was calculated with all 16S rDNA sequences and their closest relatives by using Olsen correction and a bootstrap resampling of 100 replicates.

Data analysis with UniFrac

To test differences between the bacterial communities from each sample, I used the UniFrac computational tool (Lozupone and Knight, 2005). I used the neighbor-joining tree to calculate the fraction of tree branch length unique to any one treatment in pairwise comparisons (the UniFrac metric). The p value for the tree, reflecting the probability that there are more unique branch lengths than expected by change, was calculated by generating 100 random trees. Choosing the Lineage-specific analysis option in the UniFrac computational tool I tested whether any environments are enriched for particular lineages. The lineage-specific analysis applies the G-test of significance to each lineage to determine whether the sequences have a different distribution among environments than does the tree overall. The analysis accounted for abundance information resulting from the RFLP analysis. Additionally I performed UPGMA clustering, using the weighted UniFrac metric and a jackknife analysis with 100 permutations to access confidence in nodes of the UPGMA tree.

Estimation of diversity

The estimation of the number of bacterial phylotypes in each sample was assessed by the Chao1 nonparametric richness estimator implemented in the computational tool EstimateS (Version 8, http://purl.oclc.org/estimates). For the purpose of inputting data into the program, I treated each RFLP pattern as a separate sample.

Hoechst staining and whole-cell hybridization

Mazerates were performed according to the standard protocol (David, 1973). Hybridizations of fixed mazerated *Hydra* cells (immobilized on glass slides) were done as described by Manz et al. (Manz et al., 1992) with monofluorescently labeled rRNA targeted oligonucleotide probes: EUB338 5'-GCTGCCTCCCGTAGGAGT-3' (universal eubacterial probe, positive control), and nonEUB338 5'-ACTCCTACGGGAGGCAGC-3' (EUB338 antisense probe, negative control). Probes were 5' end labeled with either fluorescein (green fluorescence) or Cy3 (red fluorescence). Hybridization were carried out by 46 °C, 90 min in followed by one wash step at 48°C, 15 min. Additionally samples were stained with Hoechst and mounted with Citifluor (Citifluor Ltd., London, United Kingdom). Examination was done by a magnification of 1,000 with a Zeiss Axioskop 2 epifluorescence microscope by using filter sets for DAPI, Cy3, and fluorescein.

Transmission Electron microscopy

For transmission electron microscopy, polyps were fixed overnight at 4 °C in 3.5% glutaraldehyde in 0.05 M cacodylate buffer pH 7.4. After removal of glutaraldehyde samples were postfixed in 0.075 M cacodylate buffer containing 1% OsO4 for 2 h at 4 °C. After washing, dehydration was carried out using ethanol, samples were incubated with 1.2 propylenoxide and embedded in Agar 100 resin (Agar Scientific, Ltd., Essex). Ultrathin sections were contrasted using uranyl acetate and lead citrate and analysed under a transmission electron microscope.

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Chapter II: Disturbing epithelial homeostasis leads to drastic changes in associated microbiota

Introduction

Microbial perturbations and imbalances at the epithelial interface underlie many human diseases, but the complexity of most natural microbe-epithelial systems has precluded detailed studies of this usually beneficial relationship. Recent studies suggest that reduced bacterial diversity in the colonic mucosa is involved in inflammatory bowel disease (Frank et al., 2007; Ott et al., 2004) and that commensal bacteria play a role in directing the development of the mammalian immune system (Mazmanian et al., 2005). But despite the realization that constant recognition of the commensal microbiota by Toll-like-receptors plays a protective role in intestinal homeostasis (Rakoff-Nahoum et al., 2004) and that a single microbial molecule, polysaccharide A, is implicated in protection from intestinal inflammatory disease (Mazmanian et al., 2008), we know little about the mechanisms and dynamics of these complex interactions. Moreover, it remains uncertain to what extent epithelial homeostasis has influence over the associated microbial community structures.

Pioneering work by Rahat and Dimentman (Rahat and Dimentman, 1982) first demonstrated that bacteria also affect growth and development in the simplest animal at the tissue grade of organization, the early-branched metazoan *Hydra*, which in the absence of bacteria has a drastically reduced budding rate. Previously, I analyzed the microbiota in different species of *Hydra* and observed, unexpectedly, that each host species supports association with a different set of bacterial phylotypes (Fraune and Bosch, 2007). These observations indicated that *Hydra* epithelia play an active role in selecting their microbiota. The findings suggest strong selective constraints operating to shape the microbial community.

Compared with various mammalian epithelia, the hydra body plan represents a simplified version: it consists of only two cell layers with a limited number of cell types. All cell types in *Hydra* are derived from only three distinct stem cell lineages, the ectodermal and endodermal epithelial stem cells and the interstitial stem cell lineage (Bosch, 2008). Interstitial stem cells

are multipotent cells located along the body column and can differentiate in a position dependent manner into nematocytes, nerve cells, gland cells and gametes (Bosch, 2007). Given this morphological simplicity and the fact that there is no physical barrier between the epithelium and the microbes, I hypothesize that *Hydra* could also be used to uncover epithelial influences on microbial community structures *in vivo*. Exploring this mutual relationship is a critical step toward understanding how host and microbe communities remain in a mutual balance after long-term co-evolution.

Results

Epithelial homeostasis can be disturbed experimentally in *Hydra magnipapillata* strain sf1

To decipher putative links between epithelial homeostasis and species-level bacterial phylotypes, I made use of mutant strain sf-1 of *Hydra magnipapillata* which has temperature-sensitive interstitial stem cells (Sugiyama and Fujisawa, 1978). Interstitial stem cells are located between the ectodermal epithelial cells and differentiate into both germline and somatic components such as nerve cells, gland cells and nematocytes (Bosch, 2008).

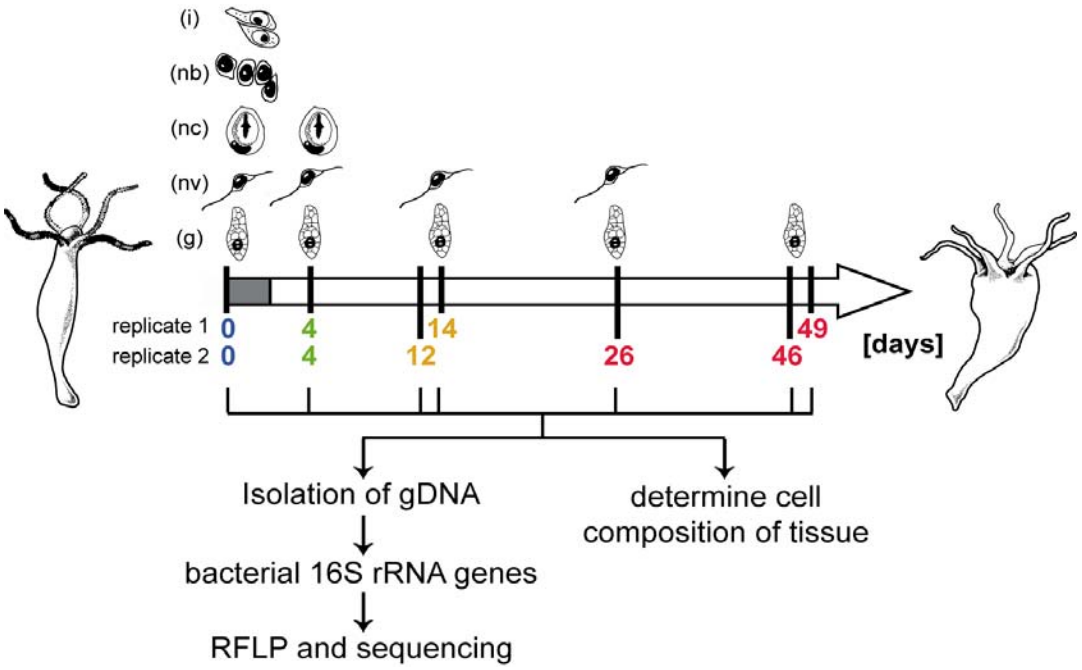

Figure 3.1. Experimental design. Animals were cultured at non-permissive temperature for 2 days prior to culturing them at 18°C. From two independent replicates polyps were collected at various time points to determine the microbiota and to monitor changes in tissue composition. Cartoons illustrate the gradual disappearance of cell types from host tissue; (i) interstitial cells, (nb) nematoblasts, (nc) nematocytes, (nv) nerve cells, (g) gland cells. Modified from (Fraune et al., submitted in revised version).

Treatment for a few hours at the restrictive temperature (28°C) induces loss of the entire interstitial cell lineage from the ectodermal epithelium while leaving both the ectodermal and the endodermal epithelial cells undisturbed (Terada et al., 1988). Following temperature treatment, the animals were cultured at the permissive temperature (18 °C) for up to 49 days

(Figure 3.1). As shown in Figure 3.2, overall morphology and integrity of the animal body remains unaffected by the temperature treatment.

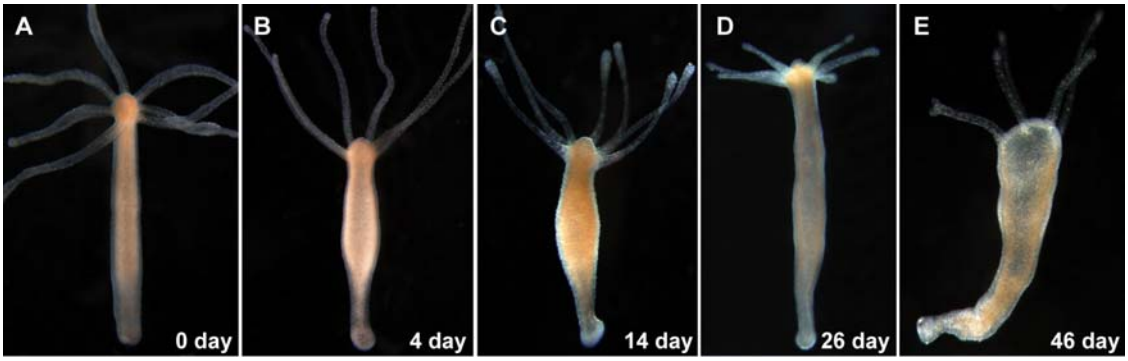

Figure 3.2. Live-images of *Hydra magnipapillata* sf-1 mutant at various time points after temperature-treatment. (A-E) As described (Sugiyama and Fujisawa, 1978), the epithelium is not affected by the temperature treatment. Modified from (Fraune et al., submitted in revised version)

Analysis of the cellular composition (Figure 3.3) clearly indicates the gradual disappearance of the entire interstitial cell lineage including interstitial stem cells, nematocytes, neurons and gland cells.

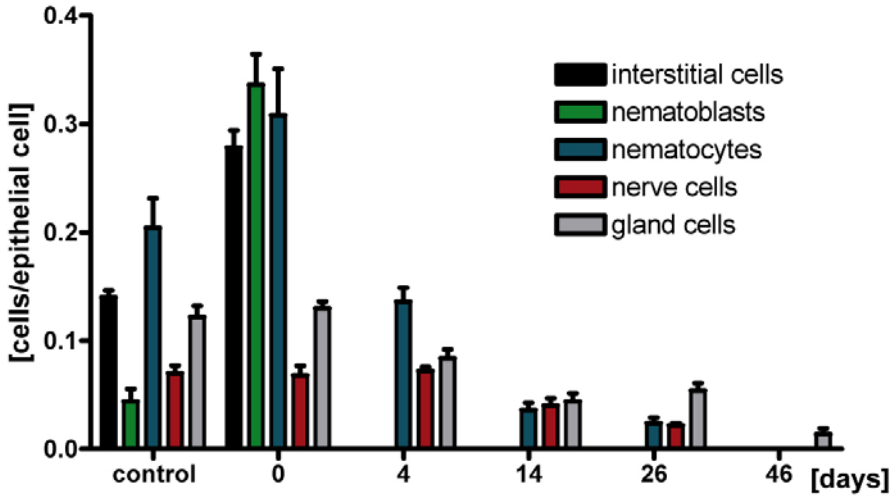

Figure 3.3. Cellular composition of polyps at different time points after temperature treatment. Note the absence of nearly all cells of the interstitial cell lineage 26-46 days after temperature treatment began. Control, cellular composition of animals kept at 18°C but starved for 40 days. Note that in control no change in nerve / epithelial and gland cell / epithelial cell ratios after long-term starvation occurs. Due to the absence of prey, in these starved control animals the number of nematoblasts is about a tenth that in well-fed controls (day 0). Modified from (Fraune et al., submitted in revised version).

Approximately 30 to 40 days after temperature treatment, the tissue nearly exclusively consists of endodermal- and ectodermal epithelial cells. Since treated polyps are unable to feed, I used

for control animals starved for up to 40 days to exclude that changes in microbiota are due to changes in metabolic activity.

Phylogenetic analysis reveals drastic differences in microbiota in control and disturbed epithelia

Next, I identified the microbiota in the hydra epithelium by performing a comprehensive bacterial 16S rRNA gene analysis at different time points after temperature treatment. The experiment was repeated two times independently with the sampling time points 0, 4, 14 and 49 days after temperature treatment in the first replicate and with the sampling time points 0, 4, 12, 26, 46 days after temperature treatment in the second replicate (Figure 3.1).

Table 1. Bacterial species richness in *Hydra magnipapillata* sf-1 mutant

	N	S	Chao 1	
			mean ± SD	95% CI
0-1	42	8	10 ± 3	(8 - 23)
0-2	40	5	8 ± 4	(5 - 30)
4-1	42	6	6 ± 1	(6 - 7)
4-2	40	4	3 ± 1	(3 - 4)
12-2	37	4	4 ± 1	(4 - 5)
14-1	47	8	8 ± 1	(8 - 14)
26-2	40	6	12 ± 7	(7 - 43)
46-2	44	7	7 ± 1	(7 - 13)
49-1	41	9	24 ± 13	(12 – 77)
control	40	3	3 ± 1	(3 - 4)

N, total number of analysed clones; S, identified bacterial phylotypes; Chao1, richness estimator; CI confidence interval. Modified from (Fraune et al., submitted in revised version).

For bacterial genotyping, genomic DNA was extracted from 10 animals from each sample and 16S rRNA genes were amplified by PCR and cloned. From each sample between 37 and 47 clones (Table 1) were randomly selected for restriction fragment length polymorphism. To identify the bacterial species in the epithelium, partial sequences (~1450 bp) were obtained from each RFLP type. Following the phylogenetic analyses, sequences with ≥ 97% similarity were designated as a phylotype. To characterize the bacterial community, I analyzed the microbiota based on nearest relative (as determined by BLASTn search) and phylogenetic affiliation. Figure 3.4 shows the phylogenetic tree with 59 16S rRNA gene sequences representing 26 different phylotypes identified in the whole sequence data set.

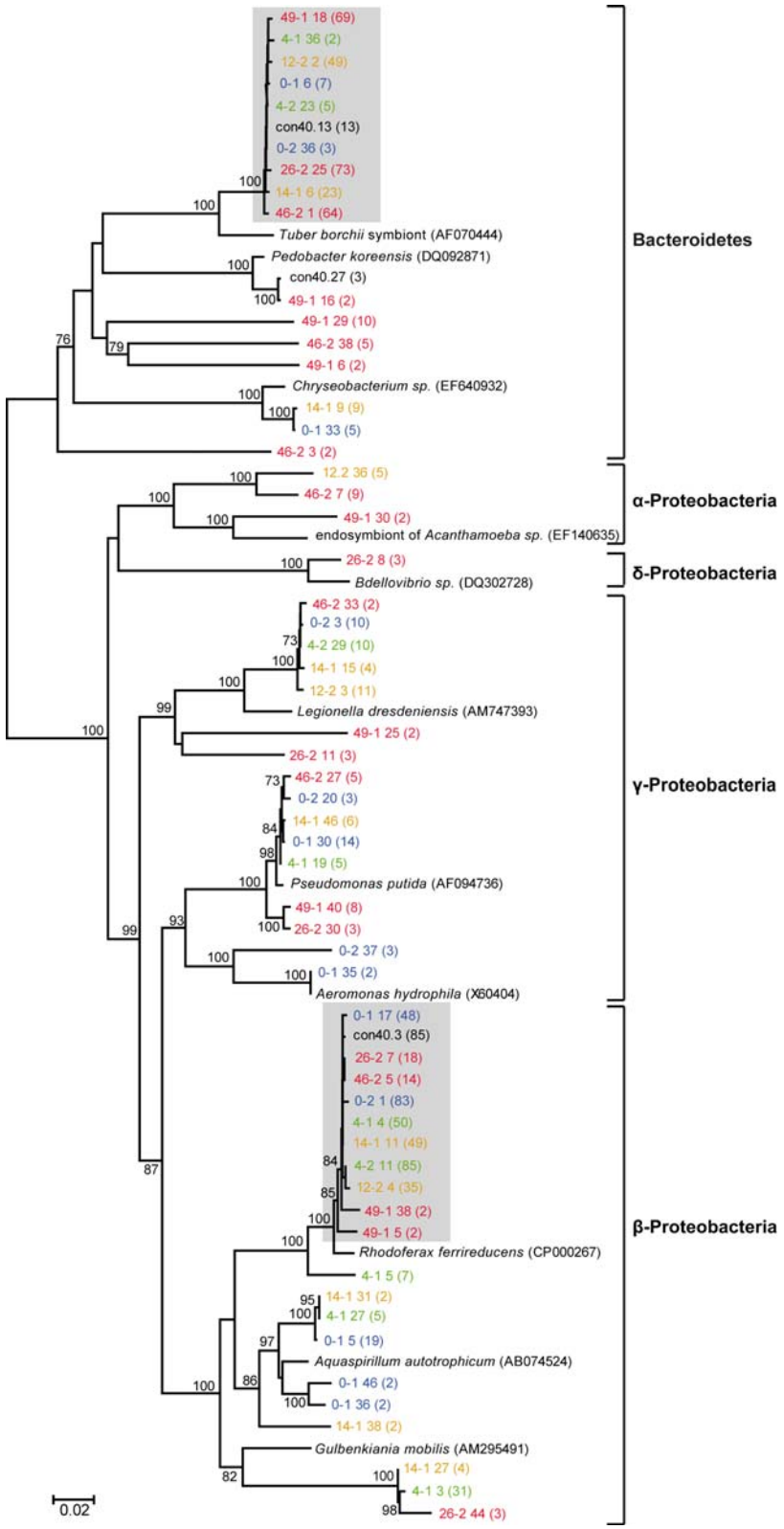

Figure 3.4. Phylogenetic positions (16S rRNA gene sequences, neighbour-joining tree) of identified bacterial phylotypes. Bootstrap values are shown at the corresponding nodes (n=1000), only bootstrap values 70 are indicated. Color of phylotypes indicates different sampling time points; blue – 0 days, green – 4 days, yellow – 12, 14 days, red - 26, 46, 49 days after temperature treatment began. Phylotypes are encoded as followed 49-1 18 (69) = 49 days after temperature treatment, replicate one, clone 18, relative abundance of this phylotype 69% in the according sample. The branch length indicator displays 0.02 substitutions per site. Light gray shadowed sequences indicate two bacterial phylotypes showing changes in their relative abundance due to the temperature treatment.

I uncovered three phylotypes in control tissue and three to nine phylotypes in the samples from the temperature treated animals (Table 1). Analyses according to rarefaction curve (Figure 3.5 A, B) and nonparametric Chao1 algorithm (Table 1, Figure 3.5 C, D) indicate that the samples shown are comprehensive. In the most samples the number of identified phylotypes corresponds to the estimated phylotype richness (Chao1). Only in the sample 49-1 the estimated number exceeded the real number of identified phylotypes (Figure 3.5 C).

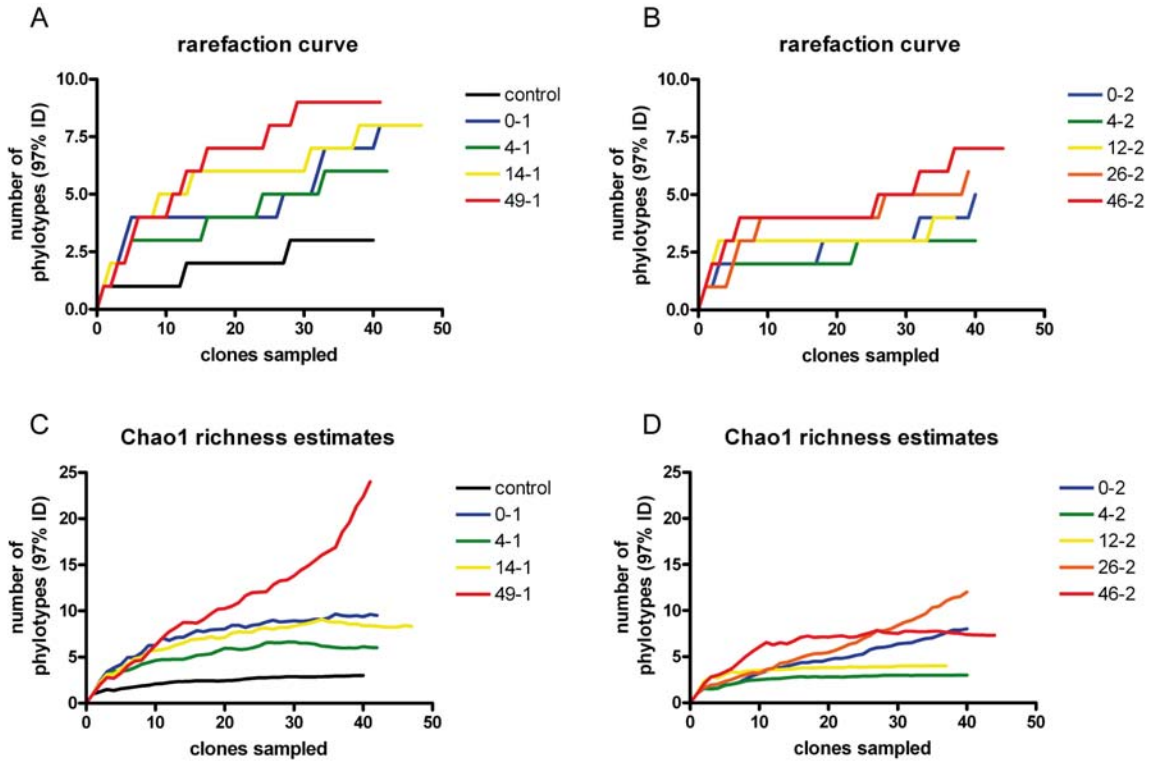

Figure 3.5. Sample-based assessments of diversity and coverage at different sampling time points after temperature treatment. (A, B) The number of observed phylotypes (97%ID) and the number of clones sampled are shown as rarefaction curves. (C, D) The phylotype richness for each treatment is expressed as Chao1 richness estimates. Modified from (Fraune et al., submitted in revised version).

The 16S rRNA sequences showed (Figure 3.4) that bacteria of only a limited number of groups (predominantly β-Proteobacteria) are present in the *Hydra magnipapillata* tissue indicating strong selection. Since *H. magnipapillata* is closely related to *H. vulgaris* (Hemmrich et al., 2007), most bacterial divisions present in the *H. magnipapillata* tissue had been documented earlier (Fraune and Bosch, 2007) when I examined the microbes in *H. vulgaris* tissue.

By identifying the bacteria species at distinct time points after temperature treatment, I next analyzed whether and how perturbations in epithelial homeostasis contribute to changes in hydra´s microbial community. Especially two bacterial phylotypes, belonging to the β-Proteobacteria and the Bacteroidetes (Figure 3.4, grey shadowed phylotypes), could be identified at all sampling time-points after temperature treatment. As shown in Figure 3.4, bacteria of the Bacteriodetes phylotype showed a 10 - 20-fold increase in relative abundance (from 7% to 69% in replicate 1 and from 3% to 64% in replicate 2), while bacteria of the β-Proteobacteria most closely related to *Rhodoferax ferrireducens* decreased in relative abundance (from 48% to 4% in replicate 1 and from 83% to 14% in replicate 2) due to the temperature treatment (Figure 3.4). To obtain additional support for the view that distinct bacterial phylotypes change in abundance with time after temperature treatment, I performed semi-quantitative PCR using phylotype-specific primers. Figure 3.6 shows that the overall abundance of eubacteria was similar in all samples taken.

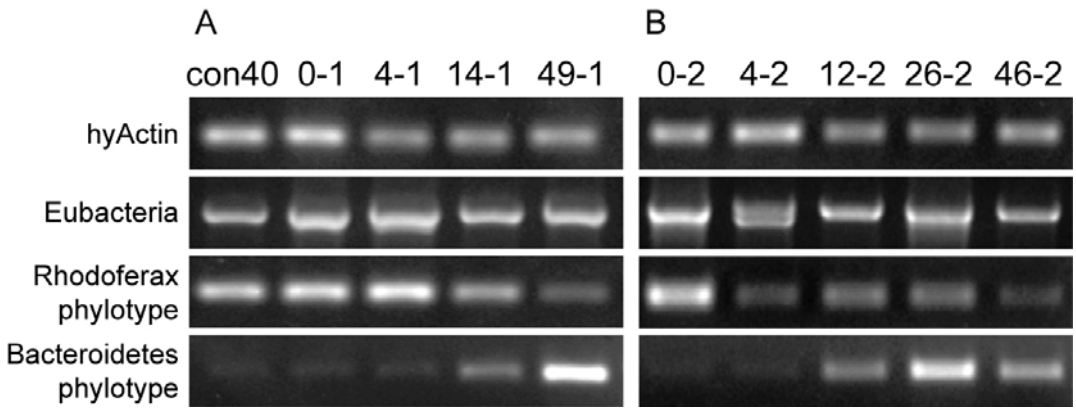

Figure 3.6. Semi-quantitative PCR with phylotype specific primers indicates drastic changes in specific phylotypes of the colonizing microbiota. (A) Semi-quantitative PCR with replicate one including control. (B) Semi-quantitative PCR with replicate two. gDNA was equilibrated using the *Hydra* actin gene. Note that the overall eubacterial load did not change due to the temperature treatment. Modified from (Fraune et al., submitted in revised version).

Intriguingly, however, about two weeks after temperature treatment I observed in both replicates a significant decrease in abundance of bacteria belonging to the Rhodoferax group concomitant with a drastic increase in abundance of the Bacteriodetes phylotype (Figure 3.6 A, B). This indicates that the composition of the colonizing microbiota is dependent on host factors such as the cell types present in the epithelium.

To summarize these changes, I analyzed the data by using the UniFrac computational tool (Lozupone and Knight, 2005) as described previously (Fraune and Bosch, 2007).

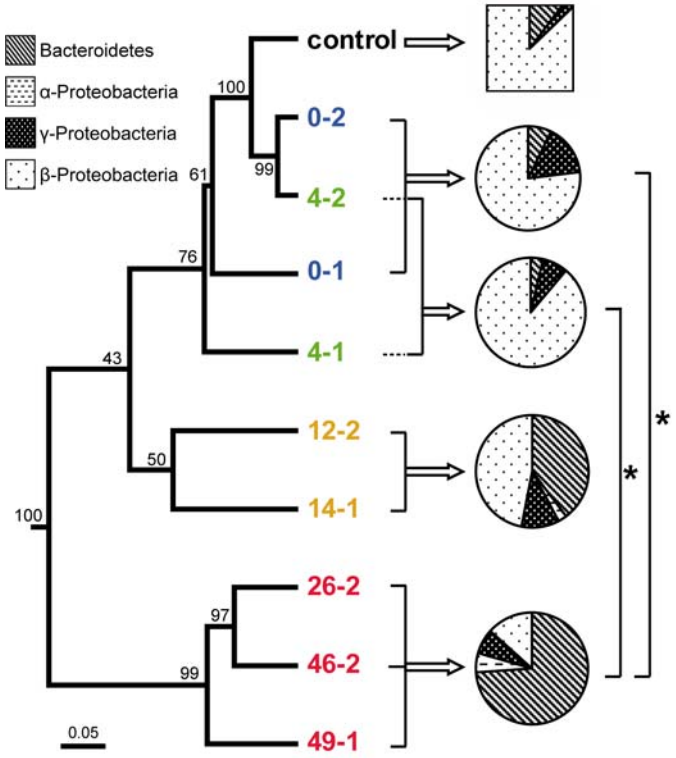

Figure 3.7. Bacterial communities change when host tissue composition changes. Jacknife environment cluster tree (weighted UniFrac metric, based on the 59 sequence tree) of the analyzed bacterial communities. Control, microbial composition of animals kept at 18°C but starved for 40 days. 1000 jacknife replicates were calculated, nodes are marked with Jacknife fractions. The branch length indicator displays distance between environments in UniFrac units. Colored numbers indicate different sampling time points (for color code see Figure 3.4). Pie charts indicate relative abundance of bacterial phyla in different samples after temperature treatment. Data from similar sampling time points of the two independent replicates were pooled. Asteriks indicate the significance of the differences in the bacterial composition (UniFrac significance $p \leq 0.05$) of the pooled data sets. Modified from (Fraune et al., submitted in revised version).

UniFrac cluster analysis allowed assignment of distinct phylotypes to each tissue sample. As shown in Figure 3.7, the bacterial composition of tissue samples from control and early time points after temperature treatment was very similar. In control tissue (i.e. not temperature treated, but starved for 40 days) as in tissue from polyps analyzed before and immediately after temperature treatment, bacteria belonging to the Rhodoferax phylotype are most abundant while bacteria of the Bacteroidetes phylotype were found only at very low relative abundance. Since interstitial stem cells and early stages of nematocyte differentiation are eliminated within two to four days after temperature treatment, these cell types apparently had no immediate influence on microbial community composition. Intriguingly, two weeks after temperature treatment, when the host tissue was lacking not only all interstitial cells as well as the

nematoblasts and most nematocytes, but in addition also had a reduced number of neurons and gland cells (see Figure 3.3), the bacterial composition changed. Most drastic changes in microbiota could be observed in polyps after about four weeks of temperature treatment compared to 0 days (p=0.04, UniFrac significance). In these polyps nearly all of the cells of the interstitial cell lineage including nematocytes, nerve cells and gland cells had disappeared. As shown in Figure 3.7, in samples from this disturbed epithelium the relative abundances of the Rhodoferax and Bacteroidetes phylotype changed drastically.

Discussion

The data presented in this study shows that changes in epithelial homeostasis in *Hydra* causes significant changes in the microbial community, implying direct interaction between epithelia and microbiota. Which factors are involved in this dynamic interplay between host and microbes? Since *Hydra* has complex epithelial cell-based mechanisms for host defence engaging novel potent antimicrobial peptides (Bosch et al., 2008; Jung et al., 2008), it is likely that these key effector molecules of the innate immune system have an essential role in this regulation. Antimicrobial peptides in *Hydra* have been found to have different antimicrobial activities against different bacteria species (Bosch et al., 2008; Jung et al., 2008). It should, therefore, not be surprising if different antimicrobial peptides affect the microbiota associated with the hydra epithelium in a different manner.

Evidence presented in this study shows that the absence of two types of interstitial cell derivatives, nerve cells and secretory gland cells, has profound impact on the composition of the colonizing microbiota. Interestingly, both cell types have been observed earlier in being involved in hydra´s microbial defence. When investigating the antibacterial activity in *Hydra*, it was observed a strong correlation between the number of neurons present and the level of the antibacterial response (Kasahara and Bosch, 2003). Using the same mutant strain of *H. magnipapillata* containing temperature-sensitive interstitial stem cells as the strain I used in this study, Kasahara and Bosch observed strong antibacterial activity in epithelial tissue lacking any neurons suggesting that in hydra neurons influence the innate immune response. Since it was probably within cnidarians, or a related ancestor group, that nervous systems first evolved (Nielsen, 2008), these observations led us to propose (Kasahara and Bosch, 2003) that the cross-talk between nervous and immune system should be considered as an evolutionary ancient invention. My observation of drastic changes in the composition of the microbiota in polyps lacking nerve cells (Figure 3.4 to Figure 3.7) underlines the significance of this cell type in host-microbe interactions.

Another cell type absent in temperature treated *H. magnipapillata* strain sf1 polyps and therefore may affecting the composition of the microbiota are secretory gland cells. Interestingly, our recent investigations show that hydra´s secretory gland cells play roles in preventing bacterial infection by producing antimicrobial mediators (Augustin et al.). Thus,

the release of immune mediators from these cells seems to be a critical early step in the complex series of cellular and molecular events which make up the innate immune response in *Hydra*.

In summary, our finding has three major implications. First, it indicates a previously unrecognized link between cellular tissue composition and microbiota. Second, the finding may be applicable to understanding mechanisms controlling host-microbe interaction in other epithelial systems. The complexity of most previously studied systems has limited detailed studies of epithelial influences on microbial distribution. Our findings derived from the *in vivo* context of a whole epithelial organism, therefore, may provide one of the simplest possible systems to address questions of how tissue composition affects the microbial community over space and time and how a stable host and microbe community remains in balance. Third, since *Hydra* is an early-branching metazoan (Collins, 1998; Collins et al., 2006) and has preserved much of the genetic complexity of the common metazoan ancestor (Hemmrich and Bosch, 2008; Miller et al., 2007), it also promises to be highly informative with respect to tracing ancient mechanisms controlling inter-kingdom interaction between bacteria and their hosts.

Material and Methods

Animal culture

Experiments were carried out with mutant strain *H. magnipapillata* sf-1 containing a temperature-sensitive interstitial cell lineage (Sugiyama and Fujisawa, 1978). Unless otherwise stated, animals were cultured using standard conditions at 18°C. Treatment of sf-1 polyps, which have temperature-sensitive interstitial cells (Terada et al., 1988), for 48h at the restrictive temperature (28°C) induces quantitative loss of the entire interstitial cell lineage including interstitial stem cells, proliferating nematoblasts and differentiating nematocytes. Cell composition and cell number in polyps at various time points after temperature treatment were determined in mazerated preparations (David, 1973).

Molecular analysis

For genomic DNA extraction, whole animals were subjected to the DNeasy Tissue Kit (Qiagen, Hilden, Germany). Universal bacterial PCR primers were used to amplify the region corresponding to positions 27 to 1492 of the *Escherichia coli* 16S rRNA gene by using a 30 cycle PCR (Weisburg et al., 1991). Resulting PCR fragments were cloned into pGEMT vector (Promega, Madison, Wisconsin) and transformed into *Escherichia coli* DH5α cells (Invitrogen, Karlsruhe, Germany). From each sample around 46 transformants were selected. Plasmid inserts were checked by PCR and subjected to restriction fragment length polymorphism by using the restriction enzymes *HaeIII* and *Hin6I* (Fermentas). By sequencing several clones, I confirmed that clones displaying an identical RFLP pattern are members of the same phylotype. Representative plasmids were sequenced using a LI-COR 4300 DNA Analyzer plate sequencer (LICOR Biosciences, Lincoln, Nebraska). All sequences have been submitted to GenBank (FJ517665 - FJ517723).

Data analysis

Sequences were sorted into phylotypes using the criterion of 97% sequence identity. All the sequences were subjected to the chimera check programs Bellerophon (Huber et al., 2004) and Pintail (Ashelford et al., 2005) for the elimination of chimeric sequences. Three sequenced could be identified containing substantial anomalies and were removed from the dataset.

Chloroplast sequences were identified and removed. The final dataset of 59 non-chimeric sequences were aligned using the ARB software package (Ludwig et al., 2004). Closely related sequences were found by the function "search for the closest relatives" implemented in the ARB software and by a BLAST search and added also to the alignment. Alignments were optimized by hand and a neighbor-joining tree was calculated with all 16S rDNA sequences and their closest relatives by using Olsen correction and a bootstrap resampling of 1000 replicates.

Semi-quantitative PCR

For two bacterial phylotypes, showing drastic changes in their relative abundance due to the temperature treatment, I designed phylotype specific primer using the computational tool Primrose 2.17 (Ashelford et al., 2002). The specific primer combinations are the following for the Rhodoferax phylotype, Rhodo_152F 5` ATA TCG GAA CGT GCC CAG TCG 3`, Rhodo_492R 5` GGA CCG TTT CGT TCC GTA CAA 3` and for the Bacteroidetes phylotype, Bacterio_700F 5` AGA GTA TAG ATG ACG TTG GCG 3` and Bacterio_1176R 5` CGC TGG TAA CTA ACA ATA GGG 3`. For amplification of eubacterial 16S rRNA genes I used the standard primer 27F and 1492R as described above. For equilibration of genomic DNA from the different sampling time points I used a primer combination amplifying the *Hydra* actin gene: hyActin34 5` AAG CTC TTC CCT CGA GAA ATC 3` and hyactin35 5` CCA AAA TAG ATC CTC CGA TCC 3`. PCR was conducted under standard conditions with annealing temperatures one Celsius degree below melting temperatures.

Data analysis with UniFrac

To test differences between the bacterial communities from each sample, I used the UniFrac computational tool (Lozupone and Knight, 2005). I used the neighbor-joining tree to calculate the fraction of tree branch length unique to any one treatment in pairwise comparisons (weigthed UniFrac metric). The p value for the tree, reflecting the probability that there are more unique branch lengths than expected by change, was calculated by generating 1000 random trees. The analysis accounted for abundance information resulting from the RFLP analysis. Additionally I performed UPGMA clustering, using the weighted UniFrac metric and

a jackknife analysis with 1000 permutations to access confidence in nodes of the UPGMA tree.

Estimation of diversity

The estimation of the number of bacterial phylotypes in each sample was assessed by the Chao1 nonparametric richness estimator implemented in the computational tool EstimateS (Version 8, http://purl.oclc.org/estimates). For the purpose of inputting data into the program, I treated each RFLP pattern as a separate sample.

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Chapter III: Maternal protection at the base of animal evolution

Introduction

At birth, babies emerge from a sterile environment into one that is laden with microbes. „Considering the importance of the fetus to our survival as a species, it is surprising that we know so little about what protects it from microbial assault“ (Zasloff, 2003). The ability of trans-generational immunity is known in many animals. In mammals, antibodies are transferred across the placenta prior birth and through breast milk postnatally (Boulinier and Staszewski, 2008). Birds, fishes and reptiles transmit passive immunity through the deposition of antibodies in eggs (Grindstaff et al., 2003). Maternal transmission of immunity has also been observed in invertebrates. In bumblebees, for example, maternal challenge has a significant effect on the total antimicrobial activity in eggs (Moret and Schmid-Hempel, 2001; Sadd and Schmid-Hempel, 2007). Nothing is known, however, about the mechanism and molecules involved in these trans-generational effects.

In *Hydra*, embryos develop outside the mother (Figure 4.1) and are directly exposed to an environment full of potential pathogens. Unlike most other cnidarians, gastrulation in *Hydra* is followed by a cuticle stage. During this stage, the embryo is surrounded by a thick protective outer layer commonly referred to as embryotheca. Thereafter, young *Hydra* polyps directly hatch from the cuticle stage (Figure 4.1).

As described earlier in the ‘General Introduction’, in the absence of physical barriers and mobile immunocytes, adult *Hydra* have an effective epithelial defense system (Bosch et al., 2008). Toll-like-receptors (TLRs) recognize pathogen associated molecular patterns (PAMPs) such as LPS or flagellin and activate potent antimicrobial peptides (AMPs). Among them is periculin, a 16 kDa protein with a characteristic cysteine pattern (Figure 4.2 A). Periculin is bactericidal, having strong activity against *Bacillus megaterium*, and is considered one of the key effector molecules of the innate immune system in *Hydra* (Bosch et al., 2008). With regard to the microbes colonizing the hydra epithelia, bacterial 16S rDNA profiling has demonstrated that hydra epithelia actively select and shape their microbiota (Chapter I) (Fraune and Bosch, 2007).

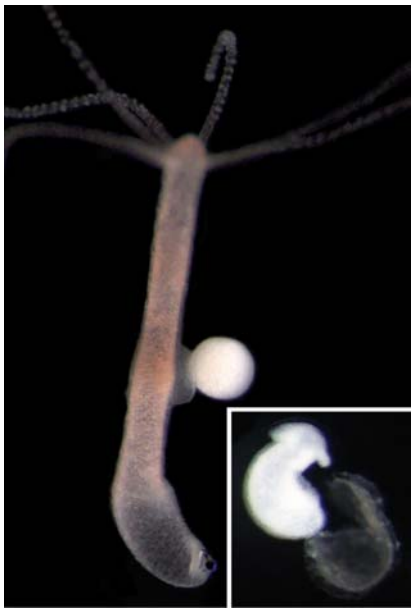

Figure 4.1. Live image of female polyp of *Hydra vulgaris* (AEP) with developing embryo, small insert: hatching polyp.

Moreover, disturbing the epithelial tissue by removing distinct cell types causes drastic changes in the microbiota (Chapter II) implying that an intimate interaction exists between bacteria and host tissue in animals at the base of metazoan evolution.

Here, I report that periculin1a is a maternally produced antimicrobial peptide, responsible for the protection of the early hydra embryo. Gain-of-function experiments using transgenic polyps ectopically expressing periculin1a in ectodermal epithelial cells allowed me to address the role of periculin1a in regulating the host-microbe interaction in detail. The reduced number of colonizing microbiota in transgenic polyps overexpressing periculin1a underlines the antimicrobial activity of this peptide. I also demonstrate a direct effect of periculin1a on the composition of the bacterial community. This represents a new role of antimicrobial peptides in regulating host-microbe homeostasis. Indirect evidence for the presence of maternally encoded antimicrobial peptides in other species, and the specificity of microbes to distinct epithelia, suggest that antimicrobial peptides may operate in a similar way in other organisms. Thus, as hypothesized earlier (Salzman et al., 2007; Wehkamp et al., 2005), antimicrobial peptides, key elements of innate immunity throughout the animal kingdom and known for their protective function, appear to have also an essential role in regulating the composition of the colonizing microbiota.

Results

Periculin, a family of amphipathic antimicrobial peptides in *Hydra*

In a suppression subtraction hybridisation (SSH) screen to identify genes involved in innate immunity of *Hydra magnipapillata* the gene encoding the antimicrobial peptide periculin1a was found (Bosch et al., 2008). Analysis of *periculin1a* transcript in the EST data bank and the corresponding genomic locus uncovered five homologues within the *periculin* gene family. Amino acid alignment (Figure 4.2 B) indicates that all five members of the *periculin* gene family encode short proteins (129–158 amino acids) with a putative signal peptide sequence at the N-terminus and a highly conserved cationic C-terminal region including 8 cysteine residues.

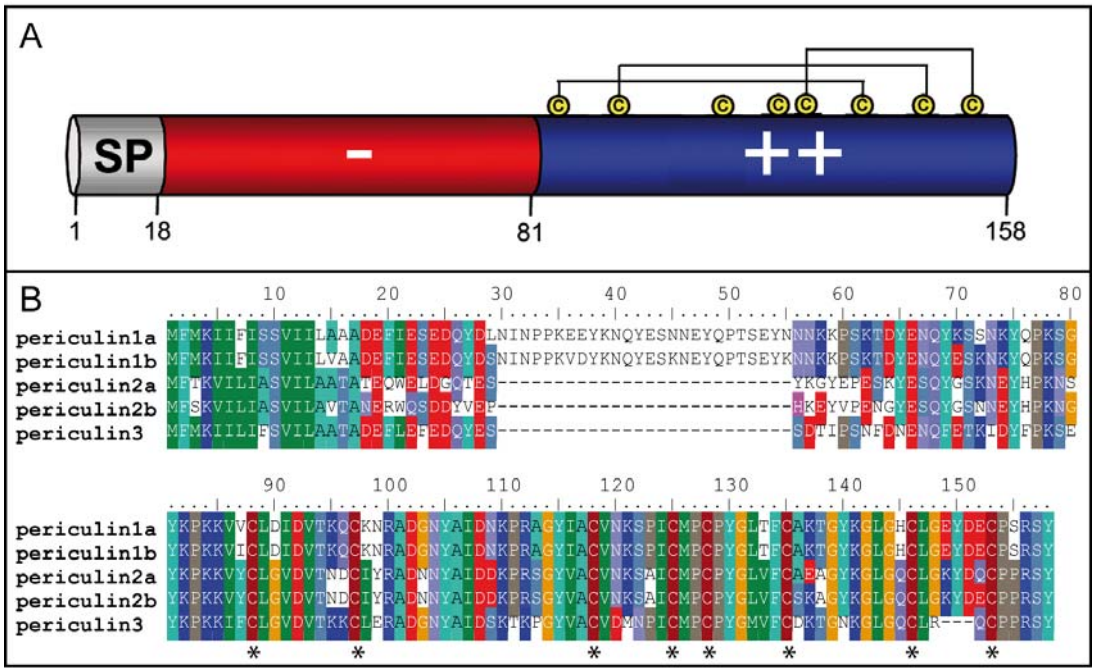

Figure 4.2. Periculin, a novel gene family in *Hydra*. (A) Schematic features of periculin genes including signal peptide sequence at the N-terminus and a highly conserved cationic C-terminal region including 8 cysteine residues. (B) Alignment of periculin peptides from the *Hydra magnipapillata* genome, stars: conserved cysteine residues.

To be able to functionally characterize the *periculin* genes in transgenic *Hydra vulgaris* (strain AEP) (Wittlieb et al., 2006), I had to isolate the *periculin* genes of *H. vulgaris* AEP. Similar to *H. magnipapillata*, *H. vulgaris* AEP contains five genes encoding periculin-like peptides. A

phylogenetic tree based on the amino acid sequence alignment (Figure 4.3) places the members of periculin peptide family in three isoform groups. The two isoforms of periculin1 and periculin2 from both *H. magnipapillata* and *H. vulgaris* (AEP) form two distinct clades, whereas periculin3 from both *H. magnipapillata* and *H. vulgaris* (AEP) forms a separate clade (Figure 4.3).

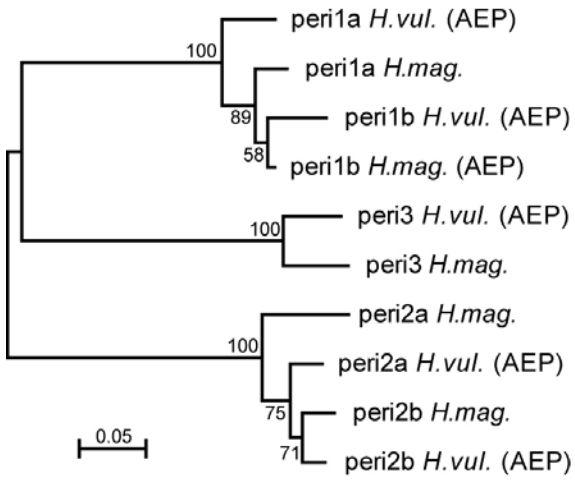

Figure 4.3. Phylogenetic analysis of periculin genes of *Hydra magnipapillata* and *Hydra vulgaris* (AEP), note that peptides from both species are orthologous.

The phylogeny clearly indicates that these genes are orthologous. According to the sequence information available, the genes of the *periculin* family have no orthologs in eukaryotic organisms outside the genus *Hydra*. They are also not present in the genome of *Nematostella*, which, as a representative of the class Anthozoa, occupies a more basal position in the phylogenetic tree than *Hydra*. Therefore, I consider the members of the *periculin* family as taxonomically restricted genes (TRGs) (Khalturin et al., 2008), which have most likely originated within the genus *Hydra*.

Periculin1a expression defines the female germ line in *Hydra*

As important effector molecule of *Hydra*´s innate immune system, periculin expression is tightly regulated and follows strict time and space constraints (Bosch et al., 2008). Consistent with our previous observation (Bosch et al., 2008), I found that in asexual polyps *periculin1a* is strongly expressed in a small population of interstitial cells within in the ectoderm (Figure 4.4 A).

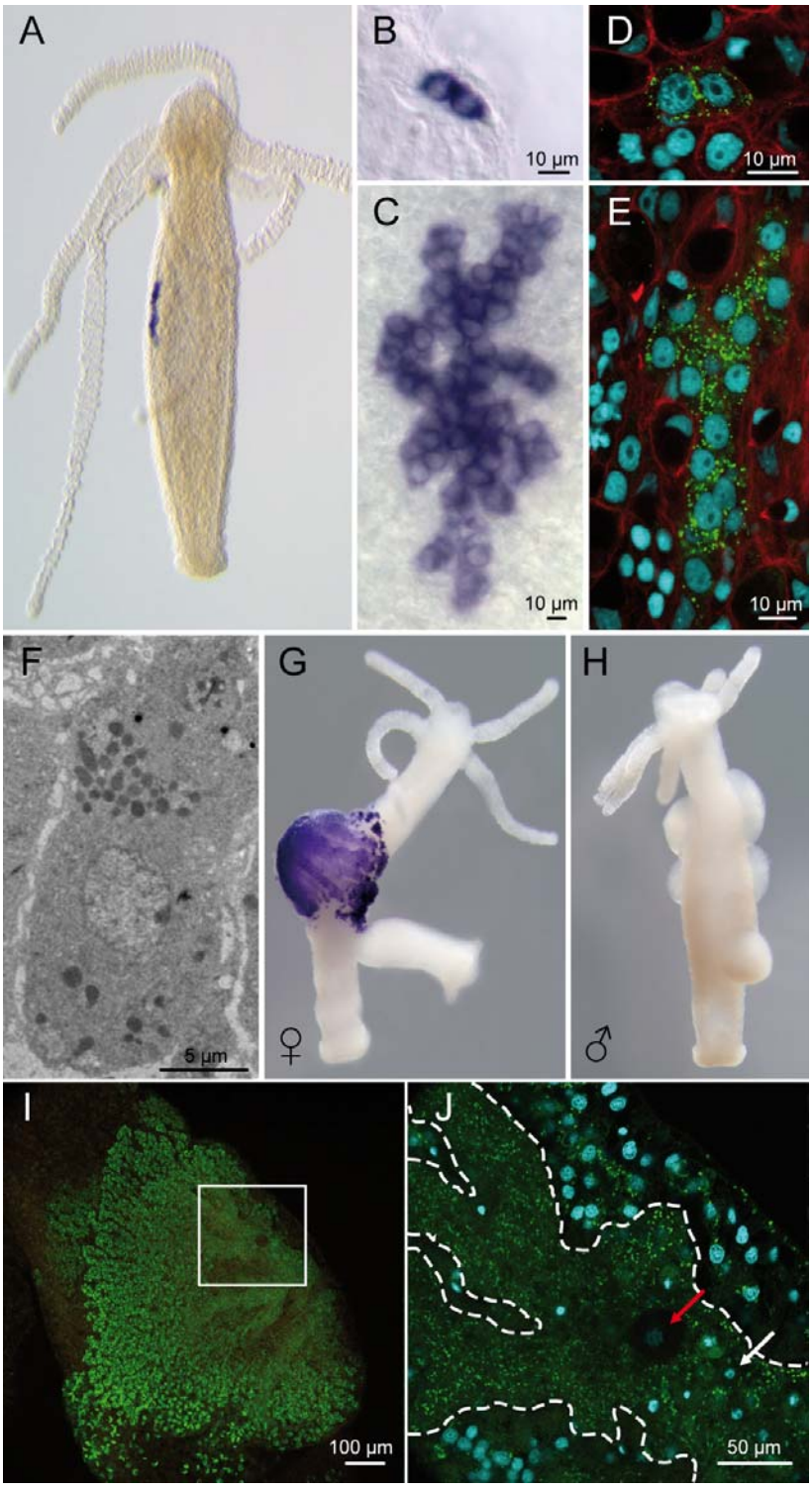

Figure 4.4. A small subpopulation of interstitial cells in the ectodermal epithelium is expressing periculin1a. (A) Low magnification view (10x) of *Hydra magnipapillata* polyp showing periculin1a positive interstitial cell cluster representing the female germline. (B) pairs of i-cells (C) cluster of i-cells. (D, E) Immunofluorescence staining of ectodermal cells for periculin showing positive vesicles in female germ line cells. (F) Transmission electron micrograph of a nurse cell containing electron dense granules in the cytoplasm. (G, H) Low magnification view (10x) of (G) *Hydra vulgaris* (AEP) female polyp showing a periculin1a expressing developing oocyte and (H) male polyp showing no signal for periculin1a. (I, J) Immunofluorescence staining of female *Hydra vulgaris* (AEP) for periculin showing positive vesicles in developing oocyte. white arrow – phagocytosed nurse cell in the ooplasm, red arrow – nucleus of the oocyte with Hoechst positive nucleolus, dashed line – cell membrane of oocyte. Green - periculin peptide; blue - HOECHST stained nucleus, red - actin filaments.

Periculin1a expressing interstitial cells are present in pairs (Figure 4.4 B) or in small clusters (Figure 4.4 C) which resemble the egg-fleck found in animals undergoing oogenesis (Honegger et al., 1989; Littlefield, 1991; Martin et al., 1997; Tardent, 1974).

No *periculin1a* expression could be detected in other cells of the interstitial stem cell lineage or in any other cell type. A antiserum, which was raised against the cationic region of periculin1a, affirmed the cell type specific localization of the periculin1a peptide. The peptide is localized within the interstitial cells in small vesicles (Figure 4.4 D, E) implying secretion. I validated this observation by an ultrastructural study of interstitial cells in the developing egg (Figure 4.4 F). There, electron-dense granules are present which appear to correspond to the antiserum positive vesicles detected by immunocytochemistry. In advanced stages of oogenesis *periculin1a* is strongly expressed in the developing egg (Figure 4.4 G). Immunofluorescence corroborates this observation and shows that periculin1a-containing vesicles are present in large number of interstitial cells recruited to form the oocyte (Figure 4.4 I).

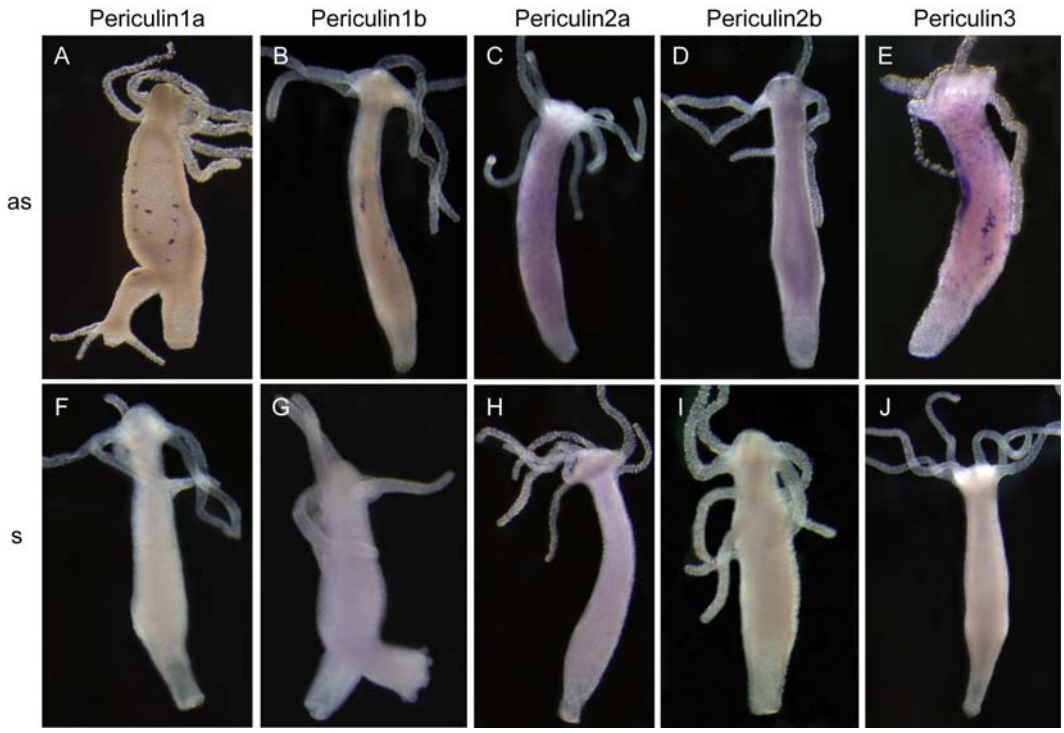

Figure 4.5. Periculin experssion in *Hydra magnipapillata*. (A-E) Low magnification view (10x) of *Hydra magnipapillata* polyps showing expression of different periculin genes. (F-J) Low magnification view (10x) of *Hydra magnipapillata* polyps showing sense control of different periculin genes.

In the mature oocyte (Figure 4.4 J), which has engulfed thousands of surrounding interstitial "nurse" cells (Miller et al., 2000), periculin1a-containing vesicles are localized within the

ooplasm. Male polyps do not express the gene (Figure 4.4 H). Thus, *periculin1a* is a maternal transcript defining the female germ line in *Hydra*. In contrast to periculin1a/b, the other members of the periculin family are not expressed in female germ line cells. In adult polyps *periculin2a/b* transcripts are present in both endodermal cells (Figure 4.5 C, D) while periculin 2a is additionally expressed in developing nematoblasts (Figure 4.6). *Periculin3* expression is restricted to endodermal cells, developing nematoblasts and a subpopulation of interstitial-cells (Figure 4.5 E).

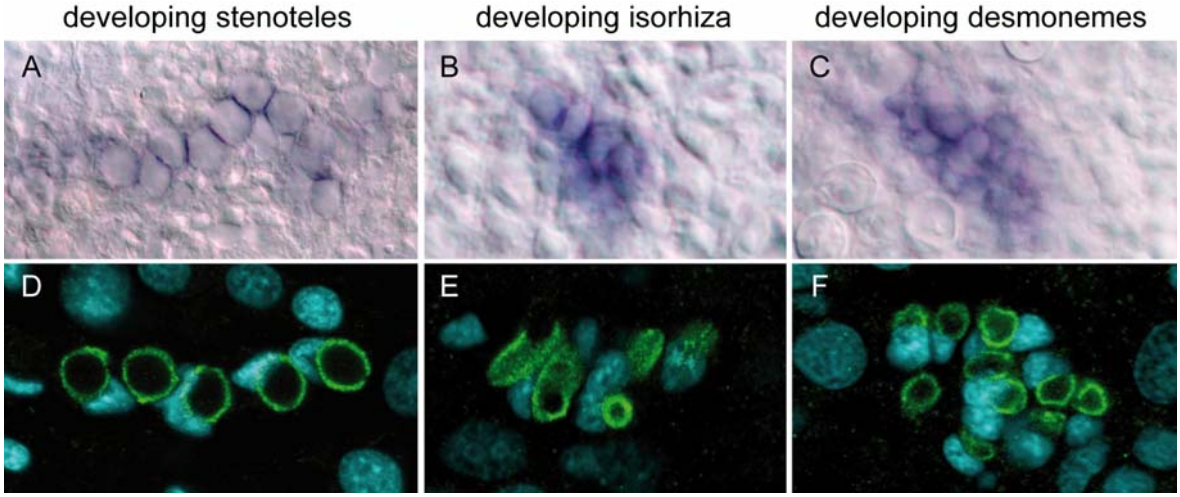

Figure 4.6. Periculin2a expression and peptide localisation in developing nematoblasts. (A-C) High magification (630 x) of showing periculin2b positive developing nematoblasts. (D-F) Immunofluorescence staining shows periculin2a peptide localisation in developing nematoblasts.

Periculin1a protects the early embryo

To assess the potential role for periculin1a in early embryogenesis, I examined both *periculin1a* expression and periculin1a peptide localization during the first cell divisions up to the gastrula stage (Figure 4.7). In situ hybridization shows a strong expression of *periculin1a* in embryos up to the blastula stage (Figure 4.7 B-F). Beginning with the gastrula stage, i.e. after midblastula transition (Figure 4.7 D, E), the in situ signal starts to become weaker indicating *periculin1a* mRNA levels at more basal levels in later stages of embryogenesis. In contrast to *perculin1a* (Figure 4.7 A), *periculin2b* is not expressed during oogenesis (Figure 4.7 H) and early stages of embryogenesis (Figure 4.7 I).

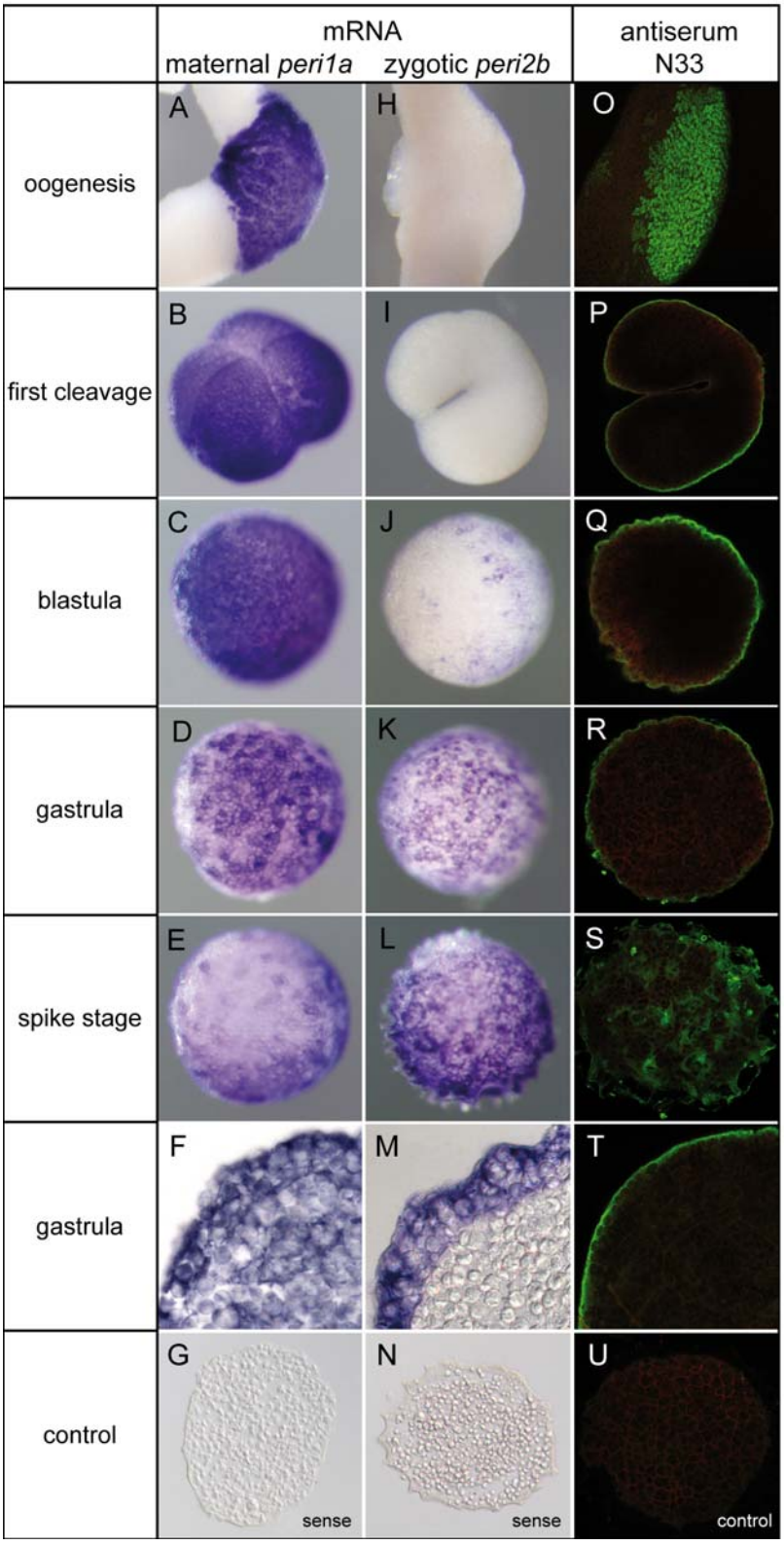

Figure 4.7. Periculin expression and peptide localization during embryogenesis (A-E) Whole mount in situ hybridisation of different embryonic stages with periculin1a probe. (F) In situ hybridisation on 12 µm section of gastrula stage with periculin1a probe (G) Sense control of periculin1a on 12 µm section of gastrula stage. (H-L) Whole mount in situ hybridisation of embryonic stages with periculin2b probe. (M) In situ hybridisation on 12 µm section of gastrula stage with periculin2b probe. (N) Sense control of periculin2b on 12 µm section of cutical stage. (O-T) Immunofluorescence staining of different embryonic stages with antiserum N33. (U) Control immunofluorescence staining with preimmune antiserum of gastrula stage. Green - periculin peptide, red - actin filaments.

After midblastula transition the blastomeres of the outer epithelial layer start to express periculin2b (Figure 4.7 J-M). To address where in the embryo the periculin peptides are located, I stained embryos at different stages with an antiserum specific for the periculin peptide family. As shown in Figure 4.7 (Figure 4.7 O to T), the periculin peptide appears to be secreted to the surface of the embryo immediately following fertilization. Upon initiation of the first cleavage (Figure 4.7 P), periculin peptide is present all over the surface of the embryo. No peptide could be detected in vesicles or in the cytoplasm of the blastomeres (Figure 4.7 O - T). After the "spike" stage and formation of the cuticle, periculin is located outside the cuticle (Figure 4.7 S). Taken together, these oberservations indicate that during embryogenesis periculin peptides are secreted directly to the surface of the outer epithelial layer.

Mature periculin is proteolytically activated during oogenesis and embryogenesis

Periculin peptides resemble prototypical antimicrobial peptides by having an anionic and a cationic region. Therefore it was predicted that periculin1a is produced as an inactive propeptide which is proteolytically activated by proteases (Bosch et al., 2008). To investigate whether during oogenesis and embryogenesis proteolytic removal of the anionic domain liberates the mature antimicrobial peptide, I used western blot analysis and tissue from adult and embryonic *H. vulgaris* AEP (Figure 4.8). As shown in Figure 4.8 A, the antiserum recognizes neither in tissue from asexual polyps nor in tissue from sexually mature males a protein. However, in protein extract from eggs, the antiserum detects two major bands of 16 kDa and 9 kDa respectively, representing most probably the propeptides of periculin1 isoforms (predicted molecular weight 16 kDa) and the released C-terminal region of these peptides. In later stages of embryogenesis the antiserum in addition to the 16 kDa and 9 kDa peptides binds to peptides of about 13 kDa. Since the antiserum does not differentiate between different members of the periculin peptide family, and since periculin2b has a predicted molecular weight of 13 kDa, this seems to reflect appearance of zygotically regulated (Figure 4.7) periculin2 isoforms. Interestingly, in total protein extract of embryos (Figure 4.8 B), the most abundant protein is a 9 kDa protein which corresponds to the released mature periculin peptides. Thus, during oogenesis and early embryogenesis proteolytic processing of the periculin prepropeptides occurs in order to release the antimicrobial 9 kDa peptides in its

active form. Defining these peptides as major components of the proteom of the early embryo (Figure 4.8 B) emphasize their significance in embryo defense responses.

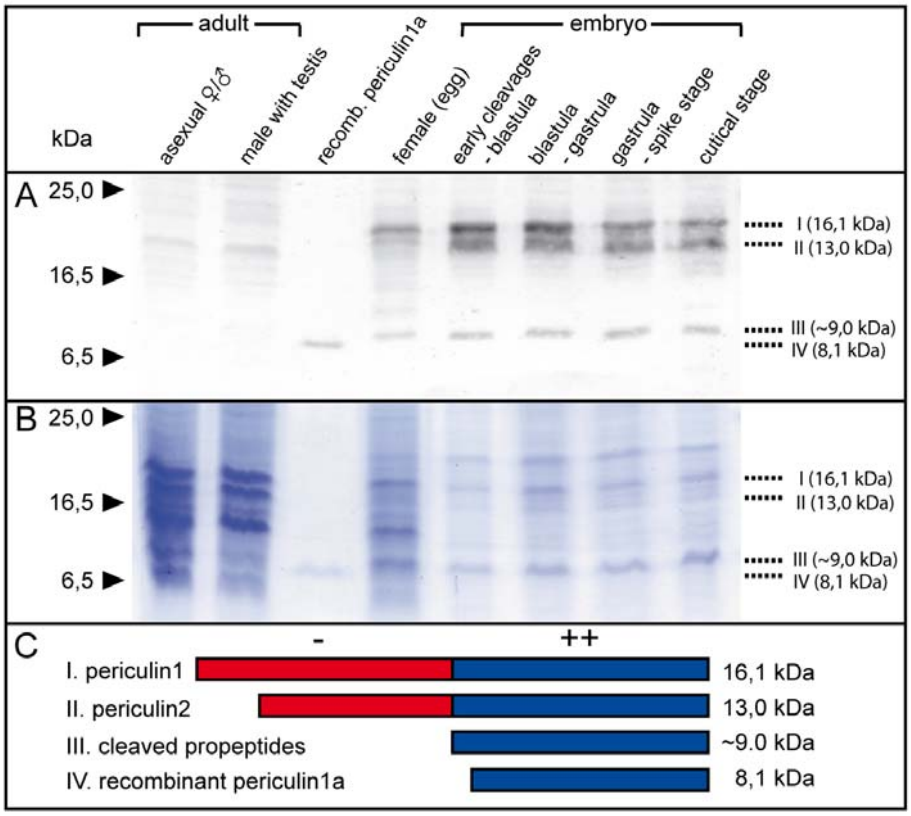

Figure 4.8. Western blot analysis of periculin expression during embryogenesis. (A) Western blot with sample of adult tissue and different stages of embryogenesis with antiserum N33. (B) Coomassie-stained SDS-page, notice, that there are only a few bands visible in the embryo samples, most prominent band corresponds to the cleaved periculin peptides. (C) Scheme of detected periculin peptides.

Transgenic *Hydra* overexpressing periculin1a in the ectodermal epithelium

To assess the biological significance of periculin1a *in vivo*, I produced transgenic polyps that overexpress periculin1a in all ectodermal epithelial cells (*H. vulgaris* (AEP) eGFP-peri1a). As shown in Figure 4.9, *Hydra* embryos were microinjected with an expression construct (Figure 4.9 A) in which two copies of full length periculin1a were fused in frame to enhanced green fluorescent protein (eGFP) and under the control of the hydra β-actin promoter. The first copy of periculin1a fused to eGFP in front of the reporter gene included the sequence encoding the signal peptide allowing *in vivo* tracing of the fusion protein; the second copy was fused behind the reporter gene and allowed normal intracellular proteolytic release of the mature peptide. As a control, transgenic line Ecto-1 (*H. vulgaris* (AEP) eGFP) was used, which

expresses the same construct, but lacks the sequences encoding for signal peptide and the periculin1a sequence (Anton-Erxleben et al., 2008).

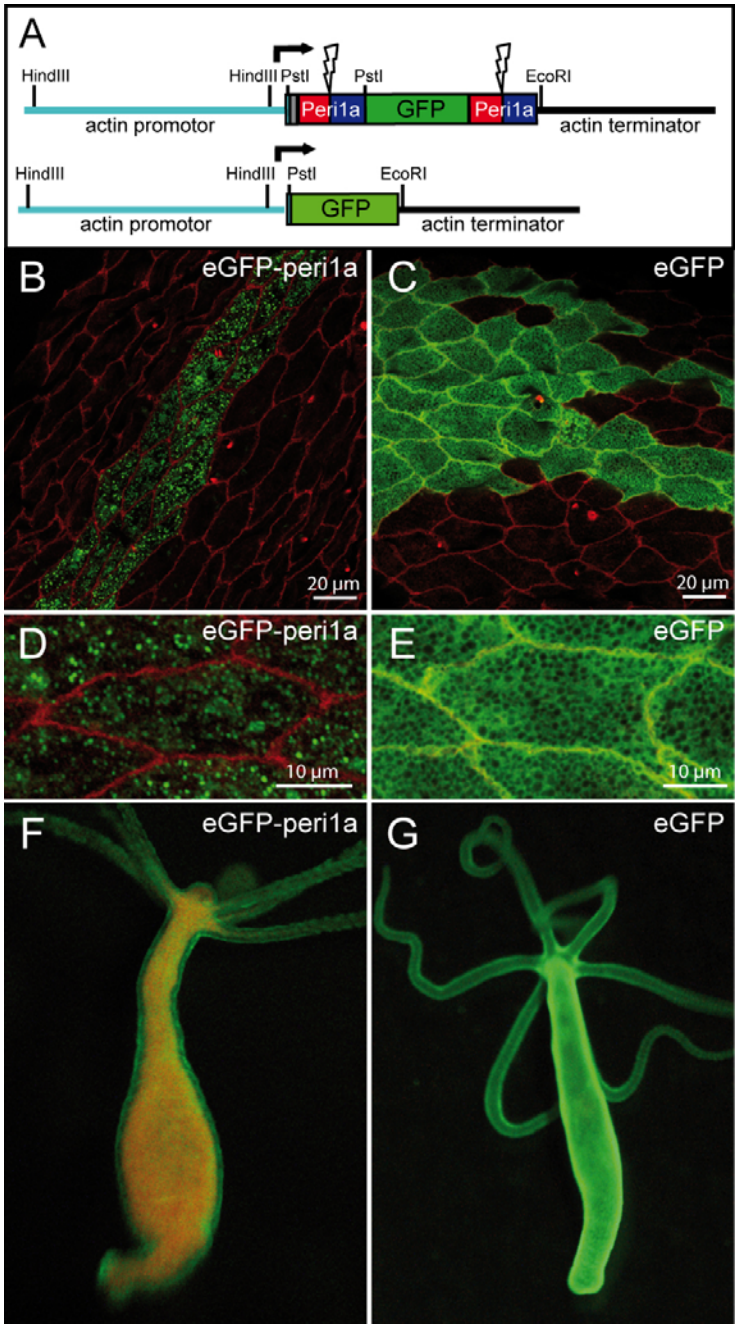

Figure 4.9. Transgenic *Hydra vulgaris* (AEP) overexpressing periculin1a. (A) Expression constructs for generation of transgenic *Hydra*. Top: Construct containing periculin1a including signalpeptide fused in frame at 5' end and periculin1a lacking signalpeptide at 3' end of eGFP. Below: Control construct with eGFP driven by 1386 bp Actin 5′ flanking region. (B, C) Confocal micrographs of ectodermal cells showing mosaic transgenic epithelium of (B) *Hydra vulgaris* (AEP) eGFP-peri1a polyp and (C) *Hydra vulgaris* (AEP) eGFP control polyp (taken from (Anton-Erxleben et al., 2008)). (D, E) Single transgenic ectodermal cells of (D) *Hydra vulgaris* (AEP) eGFP-peri1a polyp, notice peptide localization in vesicles, and (E) *Hydra vulgaris* (AEP) eGFP control polyp, notice eGFP localization in the cytoplasm. (F, G) In vivo images of transgenic polyp (F) *Hydra vulgaris* (AEP) eGFP-peri1a and (G) control polyp *Hydra vulgaris* (AEP) eGFP. Green - eGFP protein; red - actin filaments.

In transgenic founder polyps of presence of the eHFP-peri1a fusion protein was detectable in small vesicles in ectodermal epithelial cells (Figure 4.9 B, D). In control transgenic polyps, eGFP protein is localized in the cytoplasm of the ectodermal epithelial cells (Figure 4.9 C, E). Fully transgenic animals producing eGFP-peri1a in all their ectodermal epithelial cells (Figure 4.9 F) were generated by asexual propagation of mosaic founder polyps as described (Khalturin et al., 2007; Khalturin et al., 2008; Siebert et al., 2008; Wittlieb et al., 2006).

Periculin1a has direct influence on the composition of colonizing microbiota

To investigate whether periculin1a may affect the numbers and/or composition of the colonizing microbiota at the ectodermal epithelial surface, I identified the microbiota of transgenic *H. vulgaris* (AEP) eGFP-peri1a polyps and compared it with the microbiota detected in two types of control polyps, *H. vulgaris* (AEP) and transgenic *H. vulgaris* (AEP) eGFP (Figure 4.10 A).

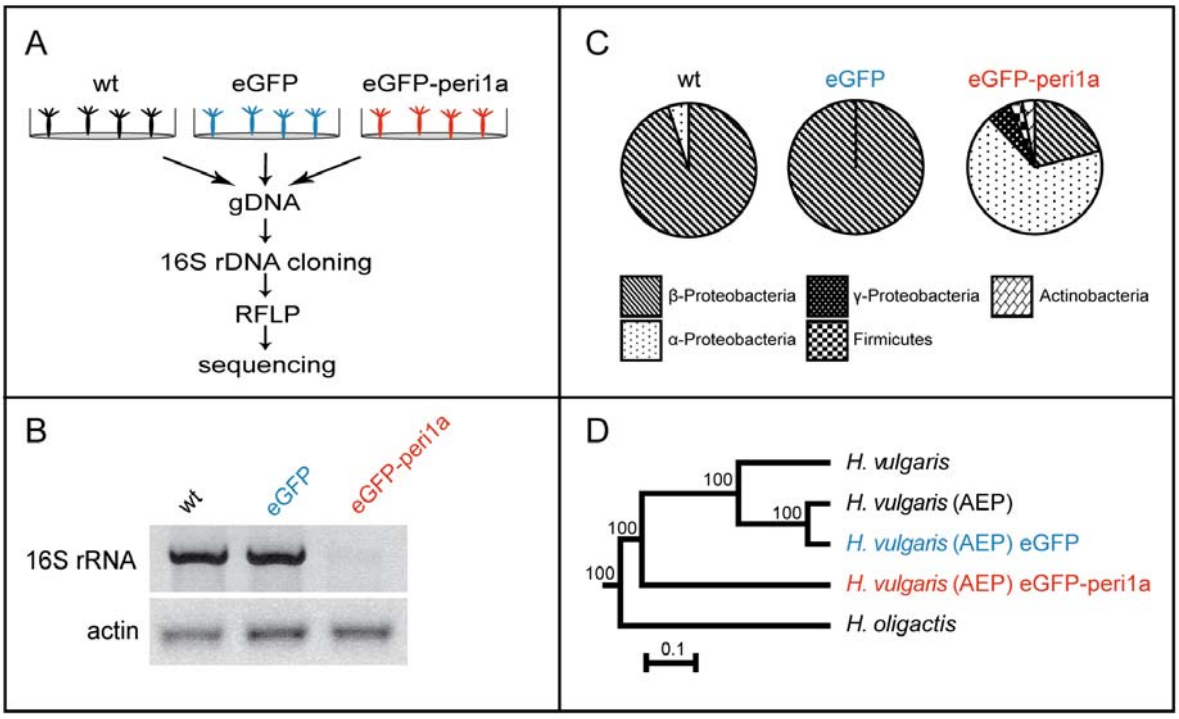

Figure 4.10. Periculin1a overexpression leads to changes in the composition of the associated microbiota. (A) Schematic representation of the approach. Bacterial compositions were compared between control polyps and polyps overexpressing periculin1a. (B) PCR on gDNA amplifying bacterial 16S rRNA genes, equilibrated on hydra actin gene. Amount on bacterial 16S rRNA genes associated with transgenic *Hydra vulgaris* (AEP) eGFP-peri1a polyps is significantly reduced. (C) Comparison of bacterial composition associated with transgenic *Hydra vulgaris* (AEP) eGFP-peri1a polyps and control polyps. (D) Jacknife environment cluster tree (weighted UniFrac metric based on a 36-sequence tree) of *Hydra*-associated bacterial communities. 1000 jacknife replicates were calculated, coverage of each node is indicated, scale bar: distance between the environments in UniFrac units.

Amplification of bacterial 16S rRNA genes revealed a significant lower bacterial load in transgenic polyps overexpressing periculin1a (Figure 4.10 B) compared to control polyps. Thus, consistent with previous observations (Bosch et al., 2008), periculin1a appears to serve protective functions by killing bacteria. To identify the composition of the colonizing microbiota, diversity analysis of bacteria was performed by amplification and cloning of 16S rRNA genes (Fraune and Bosch, 2007). From each sample, about 46 clones were randomly selected for restriction fragment length polymorphism (Fraune and Bosch, 2007). In control polyps of *H. vulgaris* (AEP) and *H. vulgaris* (AEP) eGFP I detected only one dominant RFLP pattern, while in polyps of *H. vulgaris* (AEP) eGFP-peri1a nine different RFLP patterns could be observed. To identify the bacterial phylotypes in the ectodermal epithelium, partial sequences were obtained from each RFLP type. As seen in Figure 4.10 C, control polyps are associated with only one dominant bacterial species belonging to β-Proteobacteria. The same bacterial phylotype was already detected previously (Fraune and Bosch, 2007) in association with *H. vulgaris* and *H. oligactis* (Figure 4.11). Intriguingly, I found that the composition of the microbiota which colonizes *H. vulgaris* (AEP) eGFP-peri1a polyps was drastically different from the microbiota which colonizes control polyps. As shown in Figure 4.10 C, the dominant bacterial phylotype from control *H. vulgaris* AEP polyps disappeared while in transgenics overexpressing periculin1a α-Proteobacteria and β-Proteobacteria became prevalent.

Next, I compared the bacterial communities of *H. vulgaris* (AEP), *H. vulgaris* (AEP) eGFP and *H. vulgaris* (AEP) eGFP-peri1a together with the bacterial communities from *H. vulgaris* and *H. oligactis* (see (Fraune and Bosch, 2007)) using the UniFrac computational tool (Lozupone and Knight, 2005). Intriguingly, as shown in Figure 4.10 D, the bacterial community associated with *H. vulgaris* (AEP) eGFP-peri1a clearly segregates from the communities identified in control *H. vulgaris* (AEP) and *H. vulgaris* (AEP) eGFP. Thus, the change of spatial expression of antimicrobial peptides leads to the emergence of a polyp with a novel microbiota. These results demonstrate directly and in vivo the role for antimicrobial peptide periculin1a in regulating the composition of *Hydra´*s colonizing commensals.

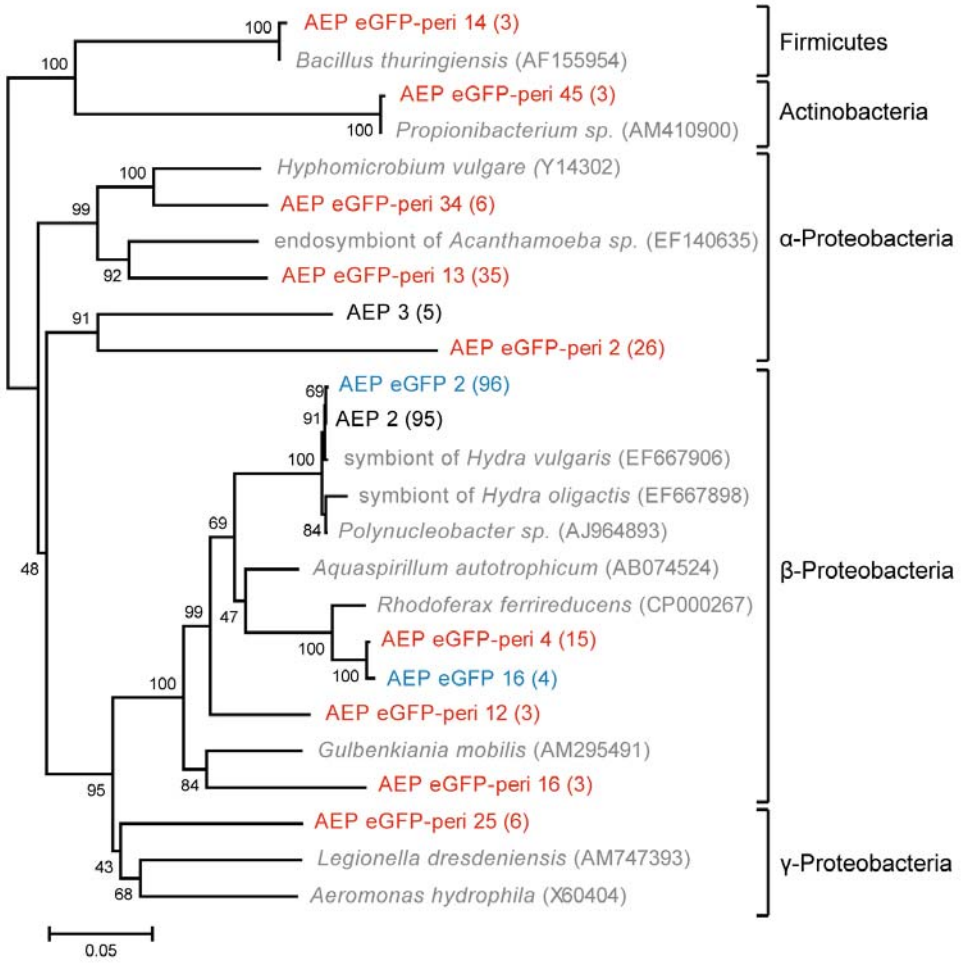

Figure 4.11. Phylogenetic positions (16S rRNA gene sequences, neighbour-joining tree) of identified bacterial phylotypes. Color of phylotypes indicate different lines of *Hydra vulgaris* (AEP). red - eGFP-peri1a, blue - eGFP, black - wt. The percentage of pylotype abundance in library is indictated in parenthesese. The branch length indicator displays 0.05 substitutions per site.

Discussion

Little is know about host factors that serve a protective function for the embryo (Zasloff, 2003) and that may regulate the composition of the colonizing microbiota (Salzman et al., 2007). I provide direct experimental evidence that in an early branching metazoan, the freshwater polyp *Hydra*, a gene-encoded antimicrobial peptide has an essential role in embryo protection, and regulates the composition of the colonizing microbes.

Here, I present the first maternally produced antimicrobial peptide which is responsible for the protection of an early embryo. Periculin1a is exclusively expressed in female germline cells and secreted in vesicles in the nurse cells of the embryo. Immediately upon fertilisation, periculin1a is released and located at the surface of the blastomeres of the developing embryo. Whether the hydra peptide is stored as propeptide similar to α-defensin 5 in human Paneth cells (Cunliffe et al., 2001) or as mature peptide similar to some α-defensins in mice (Selsted and Ouellette, 2005) is not known. After midblastula transition the transcript level of the maternally regulated *periculin1a* decreases whereas expression of the zygotically regulated *periculin2b* is induced. Therefore, the shift in the expression of the two peptides represents a shift from maternal protection to zygotic protection of the embryo. Since in immune staining of later embryo stages no positive vesicles could be observed, and since the antiserum detects both periculin1 isoforms and periculin2 isoforms, all periculin peptides appear to get directly secreted to the surface of the outer epithelial layer. Does this novel role for an antibacterial peptide operate in other organisms as well? While a precise answer to this question awaits emergence of experimental data from other animals groups, I note that in mice the cathelin-related antimicrobial peptide (CRAMP) is upregulated in neonatal intestinal epithelium and, therefore, may play a role in the protection of the newborn (Menard et al., 2008).

The diversity of microbes colonizing a given host is a result of coevolution between host and the according microbial community. Gordon and colleagues (Ley et al., 2006) suggest that the specific structure of the microbial community associated with a given host is a result of natural selection at two levels. First, competition between members of the microbiota would exert "bottom up" selection and second, the host level would represent a "top-down" selection on the microbial community (Ley et al., 2006). The innate immune system as the host's first line

of contact with the microbiota is expected to play a crucial role in this "top-down" selection of microbiota. Supporting this view, impaired host innate immune responses can directly be linked to disturbances in composition of the microbiota and to human disease. Crohn's disease and ulcerative colitis patients, for example, have abnormal gastrointestinal microbes (Frank et al., 2007); and paneth cells in patients with Crohn's disease express much lower levels of α-defensins compared to control patients (Wehkamp et al., 2005). While these observations indicate that decreased expression of antimicrobial peptides effect the associated microbiota, little is known whether and how mammalian host factors regulate the composition of this "ecosystem" (Mueller and Macpherson, 2006; Salzman et al., 2007). Here, I provide first experimental evidence that overexpression of an endogenous antimicrobial peptide not only reduces the bacterial burden but also leads to drastic changes in the biodiversity of the colonizing microbiota. Our findings indicate that antimicrobial peptides from hydra to man play a crucial role in shaping the composition of the commensal microbiota. Antimicrobial peptides, until now known as gene–encoded key elements of innate immunity, therefore, appear also to have an essential role in host-microbe coevolution.

Materials and Methods

Animals and culture conditions

Experiments were carried out with *Hydra magnipapillata* (strain 105).Transgenic experiments were carried out with animals of the *Hydra vulgaris* AEP strain as described (Wittlieb et al., 2006). All animals where cultured identically under constant environmental conditions including culture medium, food and temperature according to standard procedures.

Gene expression analysis

For assessment of gene expression, whole mount in situ hybridisation was carried out as described (Augustin et al., 2006). Probes were prepared according to the manufacturer's instructions (Roche) using sequence specific primer combinations as follows: peri1a_F: GAT GAG TTC ATA GAA TCT GAA G; peri1a_R: TCA GTA TGA ACG AGA TGG AC; peri1b_F: CAT AAA TCC GCC TAA AGT AGA T; peri1b_R: TAC TTA TTT TTA GAT TCG TAT TGG TTT; peri2a_F: AAT GGG AAT TAG ATG GTC AAA C; peri2a_R: CAA TAA ACT TTC TTT GGT TTG TAA CT; peri2b_F: GAT GGC AAT CAG ATG ATT ATG T; peri2b_R: AAT AAA CTT TCT TTG GTT TGT AAC C; peri3_F: TTT AAT TTT CTC CGT TAT TCT TGC A; peri3_R: GTT TGT ATT CAC TTT TAG GAA AG. For in situ hybridization on 12 µm thick paraffin sections, sections were prepared as described in (Frobius et al., 2003).

Genbank accession numbers of *periculin* genes

periculin1a_Hmag (FJ517724), *periculin1b_Hm*ag (FJ517725), *periculin2a_Hmag* (FJ517726), *periculin2b_Hmag* (FJ517727), *periculin3_Hmag* (FJ517728), *periculin1a_AEP* (FJ517729), *periculin1b_AEP* (FJ517730), *periculin2a_AEP* (FJ517731), *periculin2b_AEP* (FJ517732), *periculin3_AEP* (FJ517733).

Immunohistochemistry and western blot analysis

Immunohistochemistry was performed following standard procedures using paraformaldehyd fixed animals (Engel et al., 2002). Anti-serum N33 against the recombinant peptide of the

cationic region of periculin1a was raised in mice and was used in a 1/500 dilution for immune staining and 1/1000 for western blot analysis.

Confocal microscopy

Laser-scanning confocal data were acquired by using Leica TCS SP1 CLS microscope. Polyps were relaxed in 2% urethane prior to fixation in 4% paraformaldehyd. Animals were washed six times for 15 min and overnight in PBT. Following washing samples were stained with phalloidin (Fluka) and then rinsed three times for 10 min in PBT. Prior to embedding in Mowiol/DABCO, animals were incubated in HOECHST (Calbiochem).

In vivo imaging

In vivo observations were made and documented using Olympus SZX16 and Olympus DP71 digital camera.

Generation of transgenic *Hydra vulgaris* (AEP) expressing eGFP and peri1a:eGFP:peri1a in their ectodermal epithelial cells

Founder transgenic animals bearing the *actin-eGFP* construct (hotG) were produced at the University of Kiel Transgenic *Hydra* Facility (http://www.unikiel.de/zoologie/ bosch/transgenic.htm) as previously described (Wittlieb et al., 2006). For generation of *H. vulgaris* (AEP) eGFP-peri1a transgenics, a 477bp fragment of *periculin1a* coding for full-length protein including signalpeptide was amplified from *H. vulgaris* (AEP) cDNA using Platinum High Fidelity polymerase (Invitrogene) and primers Peri1_AEP_F_Pst ATG AAA ATA ATT TTT ATT TC and Peri1_AEP_R_Pst GTA TGA ACG AGA TGG AC. The cDNA was cloned into the modification of hoTG eGFP expression vector using PstI cutting site (see Figure 5 A) in front of the eGFP. The second *periculin1a* was amplified without signalpeptide and cloned into the expression vector using EcoRI cutting sites at the 3'-end of the eGFP (see Figure 5 A). The resulting transfection construct was sequenced, plasmid DNA was purified using Qiagen MidiPrep Kit and injected into *H. vulgaris* (AEP) embryos as described earlier (Wittlieb et al., 2006). Embryos began to express the reporter gene 2–3 days after injection. Founder transgenic animals bearing the *peri1a:eGFP:peri1a* construct started to hatch 16 days after microinjection. One of them showed stable integration of fusion protein in

ectodermal cell lineage. Initial founder transgenic animals were further expanded into a mass culture by clonal propagation.

Molecular analysis of associated bacteria

The whole animals were subjected to genomic DNA extraction using the DNeasy Tissue Kit (Qiagen, Hilden, Germany), according to manufactory protocol. From the gDNA bacterial 16S RNA genes were amplified by PCR with the primers 27F (5'-TG(A/G)GTTTGATC(A/C)TGGCT(C/T)AG-3') and 1492R (5'-TGG(A/C/T)TACCTTGTTACGACTT-3'). PCR was conducted with 2.5 U Taq-DNA Polymerase (GE Healthcare) and its supplemented buffer system, 1mM of each primer, 0.1 mM of each dNTP and 1 µl of extracted DNA in a final volume of 50µl under a temperature profile of 94°C for 3 min followed by 30 cycles of 94°C for 30s, 53°C for 30s, and 72°C for 1 min 40s. Resulting PCR fragments were cloned into pGEMT vector (Promega, Madison, Wisconsin) and transformed into electrocompetent DH5α *Escherichia coli* cells (Invitrogen, Karlsruhe, Germany). The plasmid inserts were checked by PCR with the vector specific primers SP6 (5' ATT TAG GTG ACA CTA TAG AAT AC 3') and T7 (5' TAA TAC GAC TCA CTA TAG GG 3') for the correct product size (approximately 1600bp). The amplified inserts of the 16S rRNA genes were subjected to restriction fragment length polymorphism by using the restriction enzymes HaeIII and Hin6I (Fermentas). Representative plasmids were sequenced using a ABI3730 capillary sequencer (ABI, Foster City, CA, USA). All sequences have been submitted to GenBank (FJ517734 -FJ517746).

Molecular phylogenetic analysis

Sequences were sorted into phylotypes using the criterion of 97% sequence identity. All the sequences were subjected to the Check chimera program Bellerophon (Huber et al., 2004) and RDP II Chimera Check (Cole et al., 2003) for the elimination of chimeric sequences. Chloroplast sequences were identified and removed. The final dataset of 13 sequences were aligned using the ARB software package (Ludwig et al., 2004). Closely related sequences were found by the function 'search for the closest relatives' implemented in the ARB software and by a BLAST search and added also to the alignment. Alignments were optimized by hand and

a neighbor-joining tree was calculated with all 16S rDNA sequences and their closest relatives by using Olsen correction.

Data analysis with UniFrac

To test differences between the bacterial communities from each sample, I used the UniFrac computational tool (Lozupone and Knight, 2005). I used a neighbor-joining tree compaining the 16S rDNA sequence data from this study with data from *Hydra vulgaris* and *Hydra oligactis* (Fraune and Bosch, 2007) to calculate the fraction of tree branch length unique to any one treatment in pairwise comparisons (the UniFrac metric). The analysis accounted for abundance information resulting from the RFLP analysis. I performed UPGMA clustering, using the weighted UniFrac metric and a jackknife analysis with 1000 permutations to access confidence in nodes of the UPGMA tree.

Transmission Electron microscopy

Polyps were relaxed in 2% urethane prior to fixation in 3.5% glutaraldehyde in 0.05 mol l–1 cacodylate buffer, pH 7.4, for 18 h at 4 °C. After washing with 0.075 mol l–1 cacodylate buffer for 30 min, animals were postfixed with 1% OsO4 in 0.075 mol l–1 cacodylate buffer for 2 h at 4 °C. After additional washing for 30 min, the tissue was dehydrated in ethanol and embedded in Agar 100 resin(Agar Scientific, Ltd., Essex). Ultrathin sections were contrasted with 2.5% uranylacetate for 5 min and lead citrate solution (prepared freshly from lead acetate and sodium citrate) for 2 min and analyzed using a transmission electron microscope CM10 or EM 208 S (Philips).

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General Discussion

Towards understanding the hydra holobiont

The aim of this thesis was to get a deeper understanding of the hydra holobiont. Formerly, it could be shown for the hydra holobiont that bacteria have an influence on the cell proliferation of the host (Rahat and Dimentman, 1982) (Figure 5.1). Under sterile conditions polyps have a reduced budding rate which can be rescued by the inoculation of the medium with bacteria. At the beginning, I focused on the identification of the associated bacteria. I could show that different *Hydra* species are associated with different microbiota, reflecting specific holobionts. Later, host factors, involved in controlling and maintaining the specific association, and the influence of the environment on the holobiont were of special interest.

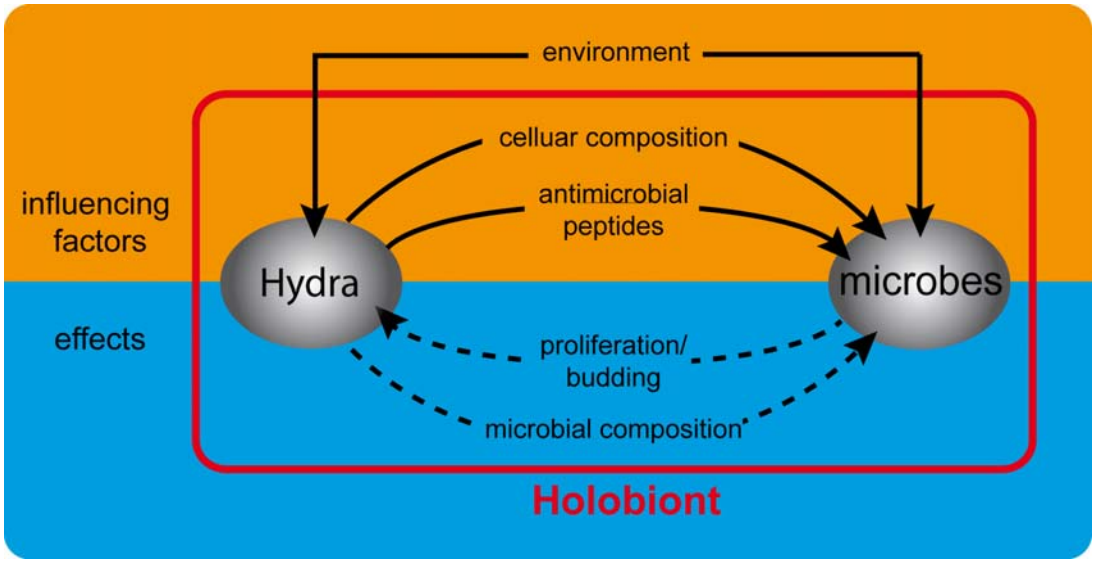

Figure 5.1. Schematic model of the hydra holobiont.

I demonstrated that both gland and nerve cells are involved in maintaining the holobiont; polyps lacking these cell types are associated with a microbiota which is drastically changed compared to control polyps (Figure 5.1). Additionally, innate immunity factors, especially antimicrobial peptides, are mainly responsible for controlling the commensal microbiota (Figure 5.1). The influence of the environment on the holobiont I analysed by changing the culturing conditions. I could show that culturing of animals from the wild under laboratory have profound effects on the bacterial composition.

In the following, I will discuss the different aspects of the hydra holobiont in detail by focussing on (i) specific epithelial host-microbe interactions, (ii) effects of host tissue homeostasis on associated bacteria (iii) antimicrobial peptides as key drivers in the host-bacteria co-evolution, and (iv) the impact of the environment on the holobiont.

Specific epithelial host-microbe interactions

All epithelia in animals, exposed to the environment, appear to be colonized by microbial communities. Therefore, it becomes important to understand the general principles by which these microbial communities evolve. Thus, I focus on the following question: *Are there identifiable core microbiota associated with a given Hydra species?* In the absence of migratory phagocytic cells the epithelium of the freshwater polyp *Hydra* is remarkably well equipped to survive in an environment teeming with potential pathogens. Toll-like-receptors (TLRs), important receptors of the innate immune system in invertebrates and vertebrates, also in *Hydra* recognize pathogen associated molecular patterns (PAMPs) such as lipopolysaccaride (LPS) or flagellin and activate potent antimicrobial peptides, such as hydramacin and periculin (Bosch et al., 2008). Nevertheless, I found that hydra epithelium is colonized by a complex and dynamic community of microbes, and individuals from different species differed greatly in their microbiota (Figure 5.2) (Fraune and Bosch, 2007). The microbial community of *Hydra oligactis* is dominated by α-Proteobacteria, while the other *Hydra* species mainly harbour β-Proteobacteria. Bacteroidetes and γ-Proteobacteria could only be identified in the very similar microbial communities of *Hydra vulgaris* and *Hydra magnipapillata* (strain sf-1) (Figure 5.2). Additionally, I observed an unexpected degree of similarity between the bacterial composition in individuals of the same species of laboratory cultures and polyps directly isolated from the wild. This indicates that the identified bacteria reflect resident species rather than transient "tourists" passing through with food, water and other environmental components.

Animal– microbe interactions and the resulting co-evolution are considered to be significant drivers of animal evolution and diversification (Doebeli and Dieckmann, 2000; Leonardo and Muiru, 2003). Host-bacteria co-evolution is commonly accompanied by co-speciation, resulting in congruent phylogenetic trees of hosts and single bacterial species. We know from bacteria which are transmitted into the next host generation directly (vertically) from the host

mother to her offspring that congruent phylogeny evolve (Clark et al., 2000; Hosokawa et al., 2006). But recently, I could show, that host-bacteria co-evolution also occurs in horizontal transmitted bacterial species of terrestrial isopods (Fraune and Zimmer, 2008). The bacterial symbionts harboured in the midgut glands of their host are transmitted environmentally (Wang et al., 2007) and provide a strong host specificity (Fraune and Zimmer, 2008).

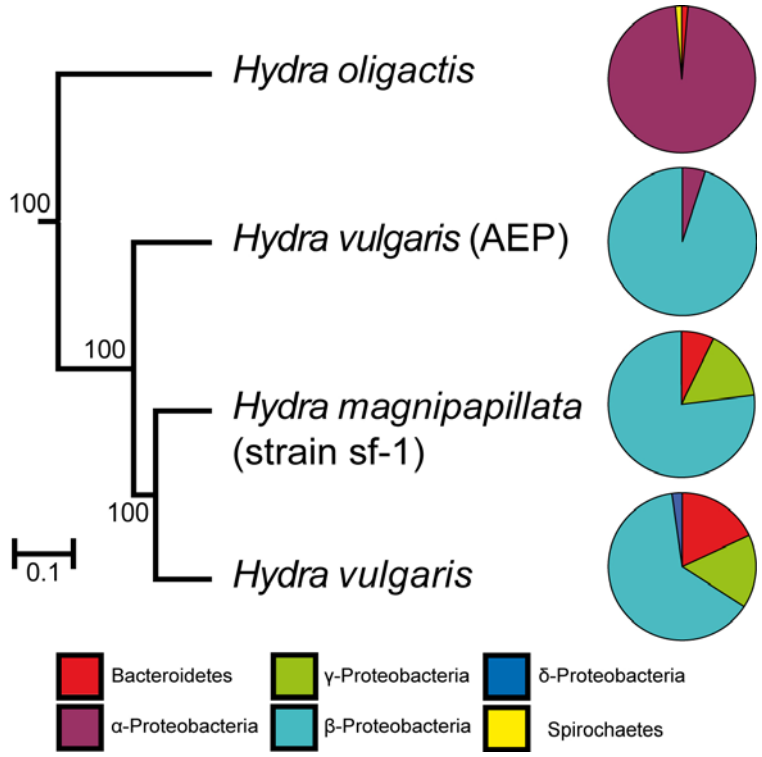

Figure 5.2. Summary of microbial communities indentified from different *Hydra* species. Data taken from Chapter I (Fraune and Bosch, 2007) (*Hydra oligactis* and *Hydra vulgaris*), Chapter II (*Hydra magnipapillata* strain sf-1), and Chapter III (*Hydra vulgaris* AEP). Jacknife environment cluster tree (weighted UniFrac metric, based on a 25-sequence tree) 1000 jacknife replicates were calculated and each node was recovered with 100%. Scale bar: distance between the different environments in UniFrac units.

But the phenomenon of congruency between phylogeny of hosts and symbionts is not limited to the single species level. Also whole bacterial communities associated with certain epithelia co-evolve with their host. For example, the fecal microbiota from 60 different mammal species co-diversified with their hosts implicating that the phylogeny has a strong effect on the bacterial community (Ley et al., 2008).

Interestingly, comparing the phylogenetic tree of the *Hydra* species (Hemmrich et al., 2007) with the according cluster tree of associated bacterial communities, they reveal a high congruency (Figure 5.3). *Hydra oligactis* as the most basal *Hydra* species analysed here is also

associated with the most distinct microbial community compared to the other microbial communities. In contrast, the close related species *Hydra vulgaris* and *Hydra magnipapillata* (strain sf-1) are associated with a very similar microbial community (Figure 5.3).

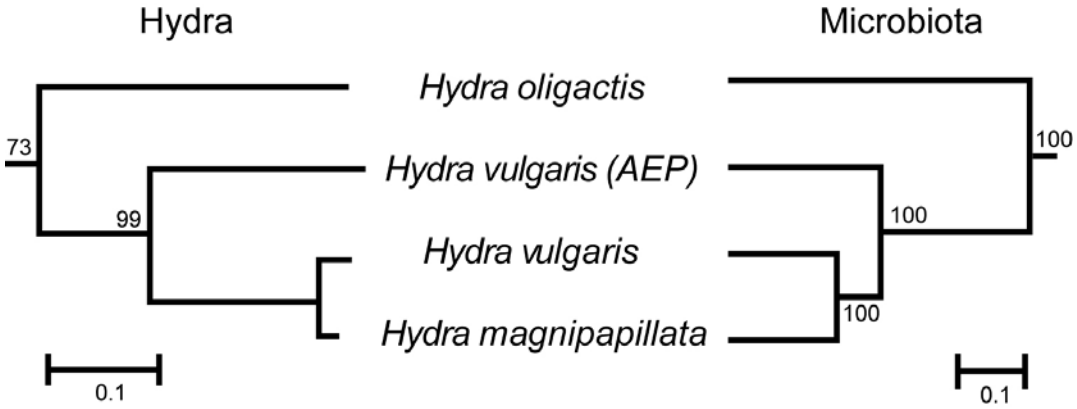

Figure 5.3. Comparison of the phylogenetic tree from *Hydra*, based on the 16S rRNA genes (modified from (Hemmrich et al., 2007), and the environmental cluster tree of the corresponding microbiota, based on the UniFrac metric (Lozupone and Knight, 2005). *Hydra* tree: Maximum likelihood phylogenetic tree inferred of the mitochondrial 16s rRNA gene Branch lengths are scaled to the expected number of substitutions. Microbiota: Jacknife environment cluster tree (weighted UniFrac metric, based on a 25-sequence tree) Scale bar: distance between the different environments in UniFrac units.

Taken together, I can say that there are specific core microbiota associated with a given *Hydra* species. The differences in the microbial communities between host species and the maintenance of specific microbial communities over long periods of time strongly indicate distinct selective pressures imposed on and within the hydra epithelium. One important factor seems to be the phylogeny of the host species, which influence the bacterial composition and diversity. Closely related host species share similar microbial communities and *vice versa* (Figure 5.3). From that I suggest that the epithelium actively selects and shapes its microbial community. The host cell types and possible host mechanisms involved in maintaining the specific holobiont will be discussed in the next sections.

But the specific association is most probably not only maintained by different host factors, but also by the bacteria itself. Bacteria are able to interfere directly with other bacteria by producing antimicrobial substances (Ritchie, 2006; Ritchie and Smith, 1995) or by producing quorum sensing molecules, like the production of N-acyl homeoserine lactone (AHL) shown in bacterial isolates from sponges (Mohamed et al., 2008). Commensal bacteria may also shape the community indirectly by competition, especially by the acquisition of limiting nutrients.

Effects of host tissue homeostasis on specific microbes

Furthermore, I ask the question: *Does disturbance of tissue homeostasis affect the associated microbiota?* To decipher putative links between epithelial homeostasis and associated species specific bacterial phylotypes, I made use of *Hydra magnipapillata* mutant strain sf-1 which has temperature-sensitive interstitial stem cells (Sugiyama and Fujisawa, 1978). In this mutant, culturing the polyps for a few hours at the restrictive temperature (28°C) induces quantitative loss of the entire interstitial cell lineage from the ectodermal epithelium while both the ectodermal and the endodermal epithelial cells were undisturbed. The overall morphology and integrity of the epithelium remains unaffected by this temperature treatment. Analysis of the cellular compositions clearly indicates the gradual disappearance of the interstitial cell lineage including interstitial stem cells, nematocytes, neurons and gland cells. Approximately 40 to 50 days after temperature treatment, the tissue nearly exclusively consists of endodermal- and ectodermal epithelial cells. These changes in the epithelia cell composition causes significant changes in the microbial community. Especially two bacterial phylotypes change drastically due to the treatment. The dominant bacterial phylotype in untreated polyps belonging to the β-Proteobacteria (Rhodoferax) decreased in treated polyps lacking the interstitial cell lineage. In contrast, a bacterial phylotype belonging to the Bacteroidetes increased in relative abundance in polyps lacking interstitial cell lineage compared to untreated and control polyps. These changes in the bacterial composition depending on the loss of interstitial cell linage alone imply a direct interaction between epithelia and microbiota.

Evidence presented in this thesis shows that especially the absence of two types of interstitial cell derivates, nerve cells and secretory gland cells, has profound impact on the composition of the colonizing microbiota. Interestingly, both cell types have been observed earlier in being involved in hydra´s microbial defence. When investigating the antibacterial activity in hydra, it could be observed a strong correlation between the number of neurons present and the level of the antibacterial activity (Kasahara and Bosch, 2003). Polyps lacking nerve cells develop a much stronger antimicrobial activity in the tissue compared to control polyps. Therefore, the authors suggest that in hydra neurons suppress the innate immune response (Kasahara and Bosch, 2003). My observation of drastic changes in the composition of the microbiota in polyps lacking interstitial cell lineage underlines the phenomena of an altered immune response due to the lack of nerve cells. Therefore, this experiment emphasizes the significance

of this cell type in host-microbe interactions and additionally may hint on the role of AMPs in shaping the commensal microbiota.

Recently, it could be also shown in *Caenorhabditis elegans* that a neural signalling functions to suppress innate immune response (Styer et al., 2008). In animals with altered neural function genes for the innate immune response were enriched. Since it was within cnidarians, where the nervous systems first evolved (Nielsen, 2008), the observation implicate that the cross-talk between nervous and immune system should be an evolutionary ancient invention (Kasahara and Bosch, 2003).

Another cell type absent in temperature treated *H. magnipapillata* strain sf1 polyps are secretory gland cells. Interestingly, recent investigations show that hydra´s secretory gland cells play a role in preventing bacterial infection by producing antimicrobial mediators (Augustin et al.). Lacking these secretory cells in the endoderm the homeostasis between host tissue and microbes in the gastric cavity is strongly effected.

To summaries, I can say that host tissue has a strong effect on the selection of associated bacteria and that distributions in the homeostasis of host tissue directly interfere with the composition of microbiota.

Antimicrobial peptides as key drivers in host-bacteria co-evolution

The diversity of microbes colonizing a host is a result of coevolution between host and the according microbial community. Gordon and colleagues (Ley et al., 2006) suggest that the specific structure of the microbial community associated with a given host is a result of natural selection at two levels. First, competition between members of the microbiota would exert "bottom up" selection and second, the host level would represent a "top-down" selection on the microbial community (Ley et al., 2006). The innate immune system is the hosts' first line of contact with the microbiota and probably plays a crucial role in this "top-down" selection of the microbiota. Indirect evidence supporting this view comes mainly from the observation, that defects in the host innate defense system affect bacterial colonization of the intestine. Crohn's disease and ulcerative colitis patients have abnormal composition of gastrointestinal microbes, characterized by the depletion of members of the phyla Firmicutes and Bacteroidetes (Frank et al., 2007), two bacterial divisions dominating the distal gut microbiota (Ley et al., 2006; Rawls et al., 2006). Interestingly, patients with Crohn's disease show also a reduced

antibacterial activity in their intestinal mucosal extracts with strongly reduced expression of paneth cell α-defensins compared to control group of patients (Wehkamp et al., 2005).

To test experimentally the hypothesis (Salzman et al., 2007) if changes in the repertoire of antimicrobial peptides in host tissue causes changes in the microbial composition in the early-brachning metazoan *Hydra* I made use of transgenic polyps. By overexpression the endogenous antimicrobial peptide periculin ectopically in ecodermal epithelial cells of *Hydra vulgaris* (AEP), I tested whether the quantity of bacterial load and/or the quality of the bacterial composition differed compared to control polyps. Intriguingly, I found, additionally to the reduced bacterial load, that the composition of the microbiota which colonizes transgenic polyps was drastically different from the microbiota which colonizes control polyps. The dominant bacterial phylotype belonging to the β-Proteobacteria from control polyps disappeared while in transgenic polyps overexpressing periculin α-Proteobacteria became more prevalent. These results provide first experimental evidence that overexpression of an endogenous antimicrobial peptide in an invertebrate host not only reduces the bacterial burden but also leads to drastic changes in the biodiversity of the colonizing microbiota. From these results I assume that specific associations between hosts and bacteria are a result of bacterial adaptation to different repertoires on AMPs in different host species. Evolutionary changes in the AMP repertoire of host species would therefore lead to changes in the composition of the associated bacterial community.

Environmental factors influencing host-microbe interactions

The association between host and microbes is also strongly effected by the environment. The fecal microbial communities from 60 mammalian species, for example, is strongly effected by their diet (Ley et al., 2008). By comparing the microbial diversity depending on the food source it could be shown that herbivore microbiota contained the most bacteria phyla, carnivores contained the fewest, and omnivores were intermediate (Ley et al., 2008). But not only the diversity was correlated with the diet, the composition of the microbiota was linked to different food sources, too. Also in invertebrate hosts the impact of the environment on associated bacteria is studied. In 2006, Rosenberg and colleagues published the "coral probiotic hypothesis" (Reshef et al., 2006). They assume that corals can adapt rapidly to changing environmental conditions by altering their associated microbiota.

To determine the impact of different environmental conditions on the bacterial community in *H. oligactis*, I cultured polyps, which were taken from the wild, for two months under standard laboratory conditions. Thereafter, I analysed the associated bacteria in comparison to the bacteria from polyps taken directly from the wild (Fraune and Bosch, 2007). Culturing of *H. oligactis* from the wild under laboratory conditions involve a change in culture temperature, culture medium and food source. These changes have significant effects on the composition of the bacterial community. One bacterial phylotype belonging to the α-Proteobacteria could be identified as the most dominant species in long term culture. The same bacterial species I identified also in polyps from the wild and two month after the shift to the laboratory, but in a lower relative abundance. Other bacterial species completely disappear from the tissue due to the change in culturing conditions (Fraune and Bosch, 2007). Thus, *H. oligactis* is not only associated with species specific bacteria but also responds to changes in the environment with changes in the bacterial community. But intra-specific differences in the bacterial composition due to changes in environmental conditions are in a smaller range than inter-specific differences (Fraune and Bosch, 2007). According to this, I assume that the hydra holobiont is a dynamic system being characterized by functional redundancy and fast adaptations to altered environmental conditions.

Outlook

Although important first steps have been made in uncovering hydra´s colonizing microbiota, much effort is need to learn more about their function. Which roles do microbial communities play in the health of *Hydra*? Does the microbial flora associated with *Hydra* contribute to the anti-microbial defense of the animal? How functionally redundant are the members of the microbiota, especially in a changed bacterial community? How does genotype, diet and health status influence the microbiota? Answers to these questions may improve our understanding of the role that tissue-associated microbial communities play in health and disease.

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Fraune S, Bosch TCG (2007) Long-term maintenance of species-specific bacterial microbiota in the basal metazoan *Hydra*. Proc. Natl. Acad. Sci. U S A 104: 13146-13151.

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Submitted

Fraune S, Abe Y, Bosch TCG Disturbing epithelial homeostasis in the metazoan *Hydra* leads to drastic changes in associated microbiota. (submitted in revised version)

Fraune S, Augustin R, Anton-Erxleben F, Klimovich VB, Samoilovich MP, Bosch TCG Maternal protection at the base of animal evolution. (submitted)

Jaenicke E, Fraune S, May S, Irmak P, Augustin R, Meesters C, Decker H, Zimmer M Phenoloxidase is not expressed during immune response in woodlice, but hemocyanin exhibits phenoloxidase activity. (submitted)

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Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation nach den Regeln guter wissenschaftlicher Praxis selbst verfasst habe. Dabei habe ich keine Hilfe, außer der wissenschaftlichen Beratung durch meinen Doktorvater Prof. Dr. Dr. h.c. Thomas C. G: Bosch in Anspruch genommen. Des Weiteren erkläre ich, dass ich noch keinen Promotionsversuch unternommen habe.

Teile dieser Arbeit wurden bereits veröffentlicht oder zur Publikation eingereicht.

Kiel, den 15.12.08

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Sebastian Fraune