

In an early branching metazoan, bacterial colonization of the embryo is controlled by maternal antimicrobial peptides

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Early embryos of many organisms develop outside the mother and are immediately confronted with myriads of potential colonizers. How these naive developmental stages control and shape the bacterial colonization is largely unknown. Here we show that early embryonic stages of the basal metazoan *Hydra* are able to control bacterial colonization by using maternal antimicrobial peptides. Antimicrobial peptides of the periculin family selecting for a specific bacterial colonization during embryogenesis are produced in the oocyte and in early embryos. If overexpressed in *hydra* ectodermal epithelial cells, periculin 1a drastically reduces the bacterial load, indicating potent antimicrobial activity. Unexpectedly, transgenic polyps also revealed that periculin, in addition to bactericidal activity, changes the structure of the bacterial community. These findings delineate a role for antimicrobial peptides both in selecting particular bacterial partners during development and as important components of a “be prepared” strategy providing transgenerational protection.

innate immunity | host-microbe interaction | embryo protection | cnidaria | *Hydra*

In many invertebrates, embryos develop outside the mother and are directly exposed to an environment full of potential pathogens. How these embryos respond to the environment-specific challenge is an interesting albeit not yet understood problem. In 1973, J. G. Wilson stated that “Living things during early developmental stages are more sensitive than at any other time in their life cycle to adverse influences in the environment” (1). This view usually is conceptualized by pointing to the absence of a sophisticated adult defense system, such as the immune system or the nervous system, and referring to parental protection based on innate mechanisms (2). Maternal factors appear to be essential for the development until the embryo utilizes its own transcriptional machinery during midblastula transition. How protection is achieved during the early developmental stages is largely unknown. In females of the basal metazoan *Hydra*, oocytes differentiate from clusters of interstitial stem cells (3–6). Following fertilization, oocytes develop outside the female into a coeloblastula (Fig. 1A). The developing embryo lacks any protective structure until post-gastrulation when the ectodermal layer extends filopodia (“spike” stage) and finally (about 24 h postfertilization) starts to form a thick protective acellular outer layer ending in the cuticle stage from which *Hydra* hatch directly (4).

Recent experimental data have indicated that, although lacking physical barriers and mobile immunocytes, adult *Hydra* possess an effective epithelial defense system (7, 8) which includes potent antimicrobial peptides (9, 10). We also have shown previously that the epithelium is colonized by a specific bacterial community (11, 12). Because disturbing the epithelial tissue by removing distinct cell types causes drastic changes in the microbiota (13), an intimate interaction appears to exist between this microbiota and the adult *Hydra* host tissue (14). In this study we examined how embryos in *Hydra* control bacterial colonization during early embryogenesis. We present data showing that the microbiota asso-

ciated with early embryos is significantly less abundant than and is very different from the microbiota of the late embryonic stages; that *Hydra* embryos have a strong antimicrobial defense system; and that maternally produced antimicrobial peptides are responsible for selecting the colonizing bacterial community.

Results and Discussion

Fewer and Different Microbes Are Associated with Early Embryos than with Later Developmental Stages. We first assessed the bacterial load of the different embryonic stages of *Hydra* by measuring cfu. Early embryonic stages were found to be significantly ($P < 0.001$) less colonized by cultivable bacteria than the transition spike stage and the cuticle stage (Fig. 1B). To identify the different bacterial communities, we performed a 16S rRNA gene analysis of the different embryonic stages (Table 1). A phylogenetic tree with the 33 different sequences represents 20 different bacterial phylotypes ($\geq 99\%$ similarity) (Fig. S1). Although the early embryonic stages (first cleavage, blastula, and gastrula stages) were colonized mainly by one bacterial phylotype closely related to *Polynucleobacter necessarius*, the spike and cuticle stages are colonized by 7 and 16 additional bacterial phylotypes belonging to the Bacteroidetes and α -, β -, γ -, and δ -Proteobacteria, respectively. To compare the bacterial communities identified from the different embryonic stages, we performed a UniFrac analysis as described previously (11). As shown in Fig. 1B, the bacterial composition of the cuticle stage segregates clearly from the early embryonic stages. Therefore, early embryonic stages seem to be capable of controlling their bacterial colonizers, whereas in later embryonic stages the cuticle appears to be a passive substrate colonized by a different and significantly more diverse bacterial community (Table 1). To confirm the different bacterial communities colonizing the different embryonic stages, we performed semiquantitative PCRs with phylotype-specific primer pairs for *Polynucleobacter* sp. (Pnec) and bacteria cultivated from cuticle stages (Table S1). Although *Polynucleobacter* sp. was present in a similar level in all analyzed embryonic stages (Fig. 1C), the bacterial phylotypes C7.1 and P1.1 representing *Curvibacter* sp. (12) were identified only in late developmental stages (Fig. 1C). The bacterial phylotypes C1.1 and

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database (periculin accession numbers: FJ517724–FJ517733; bacterial 16S rRNA genes from embryos: HQ111141–HQ111173; bacterial 16S rRNA genes from transgenic animals and controls: FJ517734–FJ517746).

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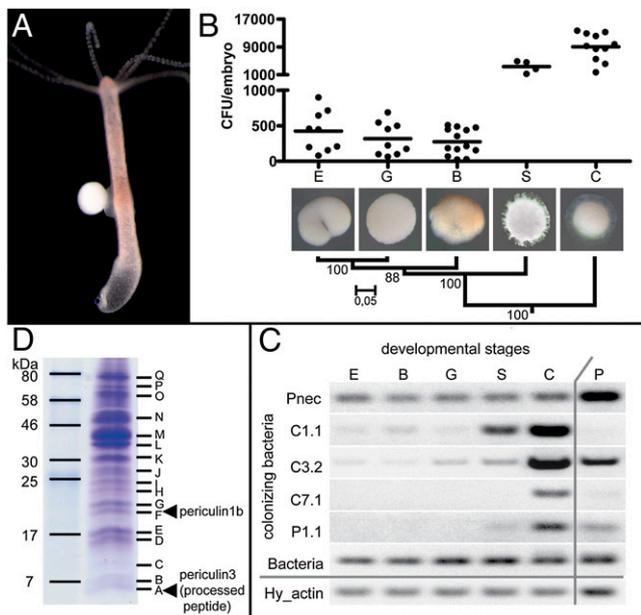


Fig. 1. Early embryos are colonized by fewer bacteria and by bacteria that differ from the bacteria that colonize later developmental stages. (A) Live image of female polyp of *Hydra vulgaris* (AEP) with developing embryo. (B) (Upper) Bacterial colonization of embryos measured by cfu per embryo. (Lower) Jackknife environment cluster tree (weighted UniFrac metric based on a 33-sequence tree) of the analyzed bacterial communities of the different embryonic stages. One thousand Jackknife replicates were calculated. Nodes are marked with Jackknife fractions. The branch-length indicator displays distance between environments in UniFrac units. (C) Semiquantitative PCR with phylotype specific primers indicates differential bacterial colonization of the different developmental stages in *Polynucleobacter* sp. (Pnec), C1.1, C3.2, C7.1, and P1.1 (bacteria cultivated from cuticle stage). (D) SDS/PAGE of protein extract of early embryonic stages used for MS analysis. In B and C, early developmental stages are abbreviated as B, blastula; C, cuticle stage; E, early cleavages; G, gastrula; P, polyp; and S, spike stage.

C3.2 were present in low abundance in the early embryonic stages, in contrast to their high abundance in the spike and cuticle stages. For two representatives we confirmed the semiquantitative data by FISH analysis. Using the phylotype-specific probe Rhodo_442, we could detect the P1.1 phylotype in small numbers on spike stages (Fig. S2A–C) and in increasing numbers on the cuticle stages (Fig. S2D–I). In contrast, *Polynucleobacter* sp. already was identifiable in early embryonic stages (Fig. S3A–D). Transmission electron microscopy revealed that *Polynucleobacter* sp. bacteria are localized within the glycocalyx of the embryo (Fig. S3E and F).

Early Embryos Possess a Well-Developed Defense System Against Bacteria. The observation that early embryos have a significantly

Table 1. Richness of bacterial species in embryonic stages of *Hydra*

Stage	N	S	Chao 1	
			Mean ± SD	95% CI
Early cleavage	21	3	4 ± 2	3–16
Blastula	22	3	3 ± 1	3–4
Gastrula	21	2	2 ± 1	2–3
Spike stage	66	8	9 ± 1	8–16
Cuticle stage	58	17	19 ± 3	17–31

Chao1, richness estimator; CI, confidence interval; N, total number of analyzed clones; S, identified bacterial phylotypes.

reduced bacterial load compared with later developmental stages (Fig. 1B) points to an effective and specific antimicrobial defense system active from the first cleavage stages on. To test the antimicrobial activity in early embryonic stages, protein extract was produced from 400 embryos, and the activity was analyzed in a minimal inhibitory concentration (MIC) assay against cultivated cuticle bacteria. Interestingly, the embryonic extract showed an MIC of 50 µg/mL against the bacteria P1.1 and of 100 µg/mL against the bacteria C3.2 and C7.1; no activity could be detected against the bacterium C1.1. Protein extract prepared from adult polyps was active against the bacterium C7.1 with an MIC of 50 µg/mL, but no activity against the three other bacteria could be detected. These data show that early embryos are capable of developing specific antimicrobial activity against certain bacterial species.

Identification of Potential Antimicrobial Peptides in the Early *Hydra* Embryo. Next, to identify potential peptides responsible for controlling bacterial colonization, we performed SDS gel electrophoresis with protein extracts of early embryos (Fig. 1D) and analyzed the protein bands of interest by MS. Eighteen different proteins were detected (Table S2). Although some of these proteins had been discovered in an earlier study designed to identify genes activated early during *Hydra*'s embryogenesis (15), two predicted peptides were similar to an antimicrobial peptide identified as component of the innate immune response in adult *Hydra* polyps (7). The protein mass fingerprint of band F corresponds to a protein similar to antimicrobial peptide periculin1 from *Hydra magnipapillata*. Protein band A also was identified as a member of the periculin peptide family. Because this peptide of ~7 kDa is smaller than the predicted size of 12.8 kDa, it appears to be the processed form of prepropeptide periculin. Identifying these peptides as major components of the proteome of early embryos emphasizes their significance in antimicrobial defense response.

Periculin, a Peptide Family of Amphipathic Antimicrobial Peptides in *Hydra*. The periculin genes encode potential amphipathic antimicrobial peptides (Fig. S4A). In *Hydra vulgaris* strain AEP, there are five periculin paralogues, referred to herein as periculin1a and -1b, periculin2a and -2b, and periculin3 (Fig. S4B). All five members of the periculin gene family encode short peptides (129–158 amino acids) with a putative signal peptide sequence at the N terminus and a highly conserved cationic C-terminal region which includes eight cysteine residues. A previous study (7) demonstrated that the cationic C-terminal region of one member of this peptide family from *Hydra magnipapillata* exhibits high bactericidal activity against *Bacillus megaterium*. A phylogenetic tree, which includes the five periculin genes from the closely related species *H. magnipapillata*, places the members of periculin peptide family in three isoform groups (Fig. S4C). Because the genes of the periculin family have no orthologs in eukaryotes outside the genus *Hydra*, they represent taxonomically restricted genes that probably originated within the genus *Hydra*.

Female *Hydra* Express Periculin1a Specifically in the Germ Line. In asexual adult polyps, periculin1a is strongly expressed in a small population of interstitial cells within the ectoderm (Fig. 2A). Periculin1a-expressing interstitial cells are present in pairs (Fig. 2D) or small clusters (Fig. 2E) which resemble the “egg-fleck” found in females undergoing oogenesis (3–6, 16). No periculin1a expression could be detected in other cells of the interstitial stem cell lineage or in any other cell type. An antiserum that was raised against the cationic region of periculin1a confirmed the cell type-specific localization of the periculin1a peptide; the peptide is localized within the interstitial cells in small granules (Fig. 2F and G), implying secretion. This observation was validated by an ultrastructural study of interstitial cells in the developing egg (Fig. 2H), where electron-dense granules were found that appear to correspond to the antiserum-positive vesicles detected by immunocytochemistry.

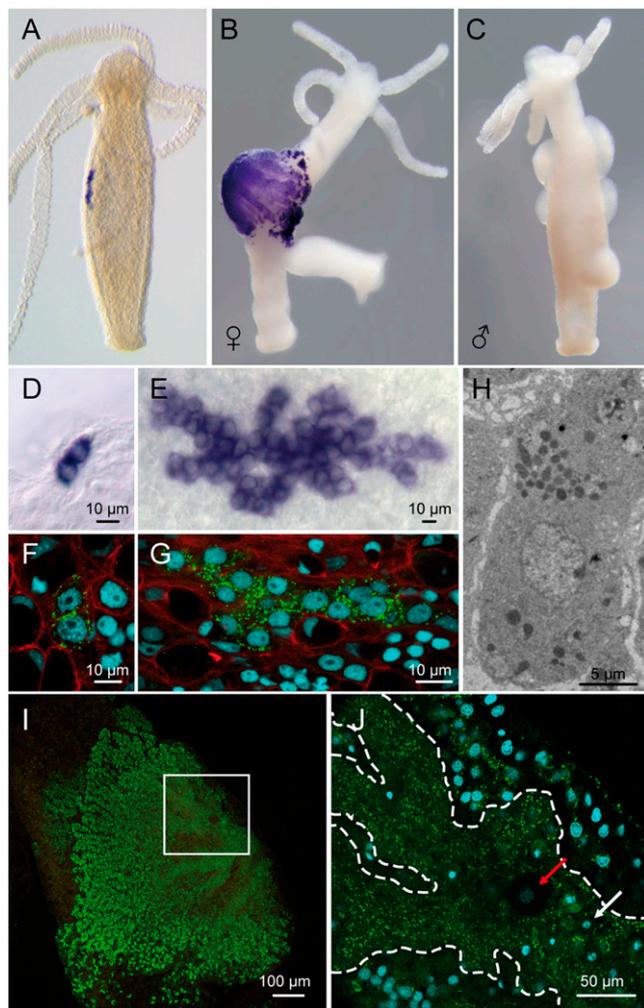


Fig. 2. Periculin1a is specifically expressed in the female germline. (A–C) Low-magnification views (10 \times) of adult polyps showing (A) periculin1a expression in asexual polyps in the female germline, (B) periculin1a expression in developing oocyte, and (C) no expression of periculin1a in male polyp. (D) Pair and (E) cluster of female germline cells expressing periculin1a. (F and G) Immunofluorescence staining of ectodermal cells for periculin showing positive vesicles in female germ-line cells: periculin peptide is green; the Hoechst-stained nucleus is blue; and phalloidin-stained actin filaments are red. (H) Transmission electron micrograph of a nurse cell containing electron-dense granules in the cytoplasm. (I and J) Immunofluorescence staining of female polyps for periculin showing positive vesicles in developing oocyte. Periculin peptide is green; the Hoechst-stained nucleus is blue; and filaments are red. J shows boxed area in I at higher magnification. The white arrow indicates phagocytosed nurse cells in the ooplasm; the red arrow indicates nucleus of the oocyte; the dashed line indicates cell membrane of oocyte.

In advanced stages of oogenesis periculin1a is strongly expressed in the developing egg (Fig. 2B). Immunofluorescence corroborated this observation and showed that granules containing periculin1a are present in a large number of interstitial cells recruited to form the oocyte (Fig. 2I). In the oocyte (Fig. 2I and J), which has engulfed thousands of surrounding interstitial “nurse” cells (17), granules containing periculin1a are localized within the ooplasm. Male polyps do not express the gene (Fig. 2C). Thus, periculin1a is a maternally regulated gene defining the female germ line in *Hydra*.

During Embryogenesis the Expression Shifts from Maternally Regulated to Zygotically Regulated Periculin Peptides. To assess the potential role for the other periculin family members in embryogenesis, both gene expression (Fig. 3 and Fig. S5) and peptide

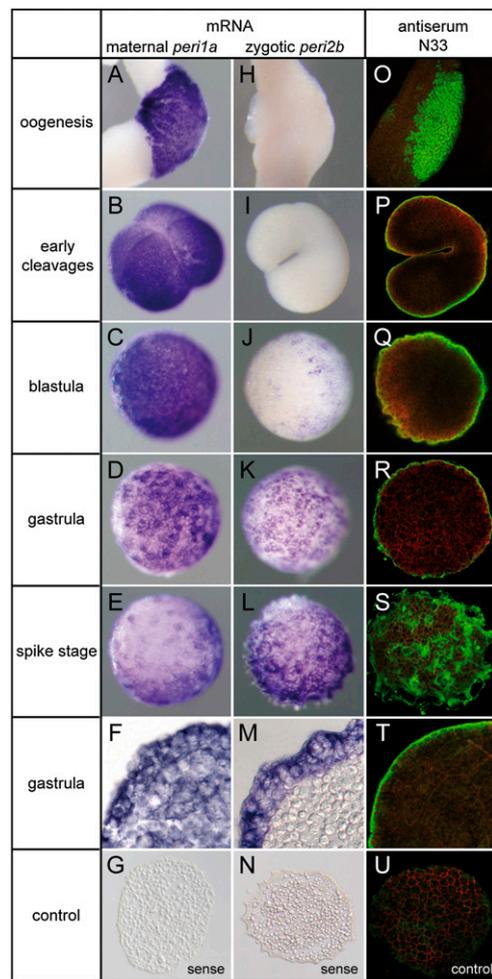


Fig. 3. Periculin expression and peptide localization during embryogenesis. (A–E) Whole-mount in situ hybridization of different embryonic stages with periculin1a probe. (F) In situ hybridization on 12- μ m section of gastrula stage with periculin1a probe. (G) Sense control of periculin1a on 12- μ m section of gastrula stage. (H–L) Whole-mount in situ hybridization of embryonic stages with periculin2b probe. (M) In situ hybridization on 12- μ m section of gastrula stage with periculin2b probe. (N) Sense control of periculin2b on 12- μ m section of cuticle stage. (O–T) Immunofluorescence staining of different embryonic stages with antiserum N33. (U) Control immunofluorescence staining with preimmune antiserum of gastrula stage. In O–U, periculin peptide is green; actin filaments are red.

localization during the first cell divisions up to the spike stage were examined (Fig. 3). In situ hybridization shows a strong expression of periculin1a in embryos up to the blastula stage (Fig. 3B and C). Beginning with the gastrula stage, i.e., after midblastula transition (Fig. 3D), the in situ signal starts to become weaker, indicating periculin1a mRNA levels at more basal levels in later stages of embryogenesis. In contrast to periculin1a (Fig. 3A), periculin2b is not expressed during oogenesis (Fig. 3H) and early stages of embryogenesis (Fig. 3I). Only after midblastula transition do the blastomeres of the outer epithelial layer start to express periculin2b (Fig. 3J–M). The expression of periculin1b and periculin3 mirrors the expression of periculin1a, whereas periculin2a mirrors the expression of periculin2b (Fig. S5). The shift in the expression within the peptide family therefore represents a shift from maternal protection to zygotic protection of the embryo. To localize the periculin peptides in the embryo, different developmental stages were exposed to periculin-specific antiserum. As shown in Fig. 3 O–T, immediately after fertilization the periculin-positive

transgenic polyps, EGFP protein is localized in the cytoplasm of the ectodermal epithelial cells (Fig. 5 C and E). Fully transgenic animals expressing EGFP:periculin1a in all their ectodermal epithelial cells (Fig. 5D) were generated by asexual propagation of mosaic founder polyps, as described previously (19–21).

To investigate whether periculin1a may affect the numbers of the colonizing microbiota at the ectodermal epithelial surface, we compared the bacterial load of transgenic *H. vulgaris* (AEP) EGFP:periculin1a polyps with two types of control polyps, *H. vulgaris* (AEP) (WT in Fig. 5F) and transgenic *H. vulgaris* (AEP) EGFP. Amplification of bacterial 16S rRNA genes revealed a significantly lower bacterial load in transgenic polyps overexpressing periculin1a (Fig. 5F) than in control polyps, suggesting that periculin1a provides a protective function by controlling colonizing bacteria. Although we expected to detect bactericidal activity, we were surprised to discover that the bacterial community structure was changed dramatically in the transgenic polyps overexpressing periculin. Analyzing the identity of the colonizing bacteria showed that the dominant β -Proteobacteria decreased in number (Fig. 5G), whereas α -Proteobacteria were more prevalent. Thus, overexpression of periculin causes not only a decrease in the number of associated microbes but also a change in the composition of the bacterial population.

Host-Derived Control Mechanisms of Bacterial Colonization in Early Development. Our data provide direct experimental evidence that in an early-branching metazoan the earliest phases of development have unique, innate mechanisms using gene-encoded maternally produced antimicrobial peptides to protect the embryo during the most sensitive phase of the life cycle. These observations have two major implications. First, embryos appear to use a “be prepared” strategy involving maternally synthesized antimicrobial peptides for protection. Second, antimicrobial peptides not only have bactericidal activity but also actively shape and select the colonizing bacterial community. Moreover, embryo-protecting peptides of the periculin family are specific for the genus *Hydra* and are not present in the genomes of other animal taxa. This specificity may reflect habitat-specific adaptations, supporting the view that taxonomically restricted host-defense molecules represent an extremely effective chemical warfare system that facilitates the disarming of taxon-specific microbial attackers (22). The natural role of the colonizing bacteria in *Hydra* is not yet clarified. The fact that the host seems to be able to select and shape the bacterial community in both adult polyps (11) and embryos may point to a complex cross-talk between host epithelium and the resident bacterial community. We note that the cuticle stage may be the only structure in *Hydra* development which, as a passive substratum for settling bacteria, is not controlled by the host.

At birth, human babies emerge from a sterile environment into one that is laden with microbes. As Zasloff (23) pointed out, “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.” In mammals, antibodies are transferred across the placenta before birth and postnatally via breast milk (24). Birds, fish, and reptiles transmit passive immunity through the deposition of antibodies in eggs (25). Because of the diversity of microbes to which they and their offspring are exposed, animals that deposit their embryos in ponds and lakes, such as amphibians and fish, would be expected to have robust, reliable antimicrobial defenses. One strategy is maternal protection. For example, the fertilization envelope of fish eggs exerts bactericidal activity (26), and the extraembryonic tissue of the tobacco hornworm is immune competent and most likely protects the embryo from infection (27). Maternal transmission of immunity also has been observed in invertebrates. In bumblebees, for example, maternal challenge has a significant effect on the total antimicrobial activity in eggs (28, 29). Our study in the early-branching metazoan *Hydra* demonstrates that maternal protection of the embryo is an an-

cestral characteristic in the animal kingdom. *Hydra*’s “be prepared” embryo-protection strategy involves a maternally encoded and environment-specific innate immune defense using antimicrobial peptides of the periculin family.

Together, the observations that early embryos have a microbiota different from that in later developmental stages and adults and that overexpression of embryonic periculin1a in adult epithelium not only reduces the microbe number but also causes changes in microbiota composition may point to a not yet fully elucidated role of antimicrobial peptides as host-derived regulators of microbial diversity. This view is in agreement with the observation that defensins in the mouse intestine act as essential regulators of the intestine microbiota (30).

Symbiont-Derived Protection in Early Development. Why *Hydra* embryos appear to select specific microbes is not known (14). However, observations in a number of aquatic animals provide hints that associated symbionts may serve a protective function for the early embryo. For example, embryos of the crustacean species *Palaemon macrodactylus* are colonized by symbiotic bacteria producing a secondary metabolite that is active against a pathogenic fungus (31). Similarly, in the salamander *Hemidactylum scutatum*, a protective bacterial community producing antifungal molecules resides in the organism’s skin (32) and can be passed directly from the mother to offspring in each generation (33). These bacteria may protect eggs and embryos from fungal infection. In the marine bryozoan *Bugula neritina* (34), a bacterial symbiont colonizes the early stages of development and produces complex polyketides, making the larvae stages unpalatable to predators (34). In squids, during spawning, bacterial symbionts secreted onto the egg jelly by the adult accessory nidamental gland may function to protect the released eggs (35, 36). To ensure the protection by these specific bacteria, host mechanisms controlling bacterial colonization, such as differential expression of antimicrobial peptides, is required.

In conclusion, we show that in an animal at a very basal evolutionary position, the earliest phases of development have sophisticated innate mechanisms to control bacterial colonization and thereby protect the developing embryo. Further analyses of the antimicrobial peptide-based protection strategy in *Hydra* will elucidate the molecular sensors by which embryos detect the microbes and also provide further insight into the evolutionary origins of present-day embryo-protection strategies.

Materials and Methods

Experimental details are provided in *SI Materials and Methods*.

Animals. Experiments were carried out using *Hydra vulgaris* (AEP strain). All animals were cultured identically under constant environmental conditions including culture medium, food, and temperature according to standard procedures.

Cultivation and Quantification of Bacteria Colonizing Hydra Embryos. Bacteria were cultivated on R2A agar plates at 18 °C for 3 d.

Bacterial Analysis by 16S rRNA Gene Sequencing. Genomic DNA was extracted and PCR was conducted using universal bacterial PCR primers (Eurofins MWG GmbH). For analysis the UniFrac (<http://bmf.colorado.edu/unifrac/index.psp>) computational tool was used (37).

Semiquantitative Analysis of Bacterial Colonization with Phylotype-Specific Primers For four cultivated bacteria and for *Polynucleobacter* sp. (Pnec), phylotype-specific primers (38) were used for semiquantitative analysis of bacterial colonization.

FISH Analysis. Hybridizations of fixed *Hydra* embryos were performed as described previously by Manz et al. (39) with monofluorescently labeled rRNA-targeted oligonucleotide probes.

Protein Extract and MIC Analysis. Protein extracts of 400 embryos and 400 polyps of *Hydra vulgaris* (AEP) were used for MIC assays, as described previously (40) with slight modifications.

Protein Gel Electrophoresis and MS Analysis. SDS gel electrophoresis was performed with protein extracts of 15 early embryos. Protein bands of interest were excised and analyzed by MS.

Gene Expression Analysis. For assessment of gene expression, whole-mount in situ hybridization was performed as described previously (41). For in situ hybridization, 12- μ m-thick paraffin sections were prepared as described (42).

Generation of Transgenic *Hydra vulgaris* (AEP) Expressing EGFP and EGFP: periculin1a in Their Ectodermal Epithelial Cells. Transgenic animals bearing the *actin-EGFP* construct (hotG) were produced at the University of Kiel Transgenic *Hydra* Facility, as previously described (21).

Immunohistochemistry and Western Blot Analysis. Immunohistochemistry was performed following standard procedures using paraformaldehyde-fixed

animals (43). Antiserum N33 against the recombinant peptide of the cationic region of periculin1a was raised in mice and was used at dilutions of 1/500 for immune staining and 1/1,000 for Western blot analysis.

Confocal Microscopy and Transmission Electron Microscopy. Laser-scanning confocal data were acquired by using Leica TCS SP1 CLS microscope, and transmission electron microscope data were analyzed using a CM10 or EM 208 S (Philips).

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