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ORIGINAL ARTICLE

Bacteria–bacteria interactions within the microbiota of the ancestral metazoan Hydra contribute to fungal resistance

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Epithelial surfaces of most animals are colonized by diverse microbial communities. Although it is generally agreed that commensal bacteria can serve beneficial functions, the processes involved are poorly understood. Here we report that in the basal metazoan *Hydra*, ectodermal epithelial cells are covered with a multilayered glycocalyx that provides a habitat for a distinctive microbial community. Removing this epithelial microbiota results in lethal infection by the filamentous fungus *Fusarium sp.* Restoring the complex microbiota in gnotobiotic polyps prevents pathogen infection. Although mono-associations with distinct members of the microbiota fail to provide full protection, additive and synergistic interactions of commensal bacteria are contributing to full fungal resistance. Our results highlight the importance of resident microbiota diversity as a protective factor against pathogen infections. Besides revealing insights into the *in vivo* function of commensal microbes in *Hydra*, our findings indicate that interactions among commensal bacteria are essential to inhibit pathogen infection.
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Introduction

In the past decade, it became evident that the epithelia of most animals are associated with complex microbial communities (McFall-Ngai *et al.*, 2013), inhabiting a broad range of body niches like the intestinal tract, oral cavity, skin, body fluids (Human Microbiome Project Consortium, 2012) or even specialized structures like bacteriocytes or cuticular pouches in insects (Douglas and Wilkinson, 1998; Currie *et al.*, 2006). In vertebrates, the gastrointestinal tract is colonized with a dense and diverse microbial community that is an important factor in health and physiology (Lozupone *et al.*, 2012; Sommer and Bäckhed, 2013). The diversity in microbiota composition and habitats is equaled by a broad variety of beneficial functions to the colonized host. The intestinal microbiota can stimulate stem cell turnover (Jones *et al.*, 2013), gut development (Rawls *et al.*, 2004) and facilitate nutrient supply by breakdown of complex carbohydrates or synthesis of essential amino acids (Sandström *et al.*, 2000; Douglas *et al.*, 2001; Yatsunenko *et al.*, 2012). Furthermore, commensal microbes are able to stimulate fundamental aspects of innate and adaptive immunity such as T-cell maturation, production of IgA, mucus secretion and induction of antimicrobial peptides (Dobber *et al.*, 1992; Mazmanian *et al.*, 2005; Weiss *et al.*, 2012).

In 1955, Bohnhoff *et al.* (1955) already demonstrated that mice with intact endogenous bacterial colonization require 100 000 times higher inocula to establish *Salmonella enterica* infection compared with streptomycin-treated mice, a mechanism known as 'colonization resistance' (Buffie and Pamer, 2013). Several mechanisms have been proposed to explain such symbiont-mediated interference with the growth of pathogens (Haine, 2008; Hamilton and Perlman, 2013). These involve exploitative competition between symbionts and pathogens for limiting factors such as nutrients (Maltby *et al.*, 2013) and adhesion receptors (Juge, 2012). Furthermore, beneficial microbes can stimulate the host's immune system against potential pathogens (Vaishnava *et al.*, 2008), a mechanism analogous to apparent competition, in which an increase in one species causes an increase in a predator that negatively affects a competitor (Holt, 1977). Third, production of microbicidal factors is a common case of interference competition among bacteria. Numerous bacteriocins produced by the intestinal microbiota are active against potential pathogens

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such as *Listeria*, *Salmonella* and *Clostridium* species (Dabard *et al.*, 2001; Gong *et al.*, 2010; Rea *et al.*, 2010). Cuticular *Streptomycetes* bacteria are protecting the offspring of digger wasps from fungal pathogens by producing a complex cocktail of antibiotics (Kroiss *et al.*, 2010). The role of individual members of a highly diverse bacterial community associated with a host remains largely unclear. In addition, little is known about the effects that interplay between commensal bacteria might have. Better mechanistic insight into the interactions among the commensal microbiota in the epithelium is thus key.

Epithelia are the first line of defense against pathogenic microorganisms. As a barrier the epithelium has to coordinate physiological functions with the control of commensal microbes and the prevention of pathogenic infections. A characteristic feature of most animal epithelial cells is a dense carbohydrate-rich layer at the apical cell surface, referred to as the glycocalyx (Ouwerkerk *et al.*, 2013). The glycocalyx represents a highly diverse and constantly renewed range of transmembrane glycoproteins, proteoglycans and glycolipids (Moran *et al.*, 2011). As it excludes large molecules and organisms from direct access to the cell surface by steric hindrance, whereas smaller molecules might pass through, the glycocalyx represents the first line of contact between host cells and bacteria and viruses.

The cnidarian *Hydra* is a useful model to characterize a barrier epithelium, innate immune responses, tissue homeostasis and host–microbe interactions (Fraune and Bosch, 2007, 2010; Bosch *et al.*, 2009; Fraune *et al.*, 2009; Franzenburg *et al.*, 2012; Bosch, 2013; Franzenburg, 2013b). Whereas polyps are colonized by a 'low-complexity' microbiota, the holobiont forms a highly specific ecosystem (Fraune and Bosch, 2007; Franzenburg *et al.*, 2013b) that is similar between laboratory-raised animals and animals being taken from the wild (Fraune and Bosch, 2007). The ectodermal and endodermal epithelium is constantly renewed and the endodermal epithelium fulfills functions similar to that of the intestinal epithelium in mammals (Augustin and Bosch, 2010). The recognition of bacteria is mediated by an intermolecular interaction of HyLRR-2 as receptor and HyTRR-1 as signal transducer (Bosch *et al.*, 2009). Upon activation, the receptor recruits the primary adaptor molecule MyD88 (myeloid differentiation factor 88). This receptor complex activation then triggers the innate immune response that involves the production of a variety of immune effector genes (Franzenburg *et al.*, 2012). Antimicrobial peptides are major components of the innate immune system of *Hydra* (Augustin *et al.*, 2009a; Bosch *et al.*, 2009; Bosch, 2013). The expression of selective antimicrobial peptides is critical for colonization by stable and species-specific bacterial communities (Fraune *et al.*, 2010; Franzenburg *et al.*, 2013b). Intriguingly, most of the antimicrobial genes identified so far are expressed in the endodermal epithelium lining the gastric cavity (Augustin *et al.*, 2009a, b; Bosch *et al.*, 2009). It remained to be shown, therefore, which mechanisms contribute to pathogen clearance in the ectodermal epithelium.

Here, we have used a gnotobiotic *Hydra* model to analyze the localization and importance of commensal bacteria in prevention of fungal infections. We identified commensal bacteria residing in the multilayered glycocalyx covering ectodermal epithelial cells. We also show that in the absence of these colonizers *Hydra* polyps are prone to fungal infection. Restoring the specific microbiota in gnotobiotic polyps prevents fungal infection. Strikingly, mono-associations with distinct members of the microbiota are not efficient or fail to provide protection. In contrast, synergistic and additive interactions of certain bacterial colonizers provide a significant resistance against *Fusarium* infections. Thus, bacteria–bacteria interactions within the commensal microbiota associated with the *Hydra* epithelium appear to be central to pathogen clearance.

Materials and methods

Animals

Experiments were carried out using *Hydra vulgaris* (AEP) (Hemmrich *et al.*, 2007). All animals were cultured under constant temperature (18 °C), light conditions (12 h/12 h light/dark rhythm) and culture medium (0.28 mM $CaCl_2$, 0.33 mM $MgSO_4$, 0.5 mM $NaHCO_3$ and 0.08 mM KCO_3) according to the standard procedure (Lenhoff and Brown, 1970). The animals were fed three times a week with first instar larvae of *A. salina*. During recolonization and experimental infection with *Fusarium sp.*, polyps were not fed.

Confocal microscopy of Hydra glycocalyx

Polyps were fixed in 2% paraformaldehyde, 2.5% glutaraldehyde and 75 mM L-lysine in 50 mM cacodylate buffer, pH 7.4, for 18 h at 4 °C. Animals were washed six times for 10 min in phosphate-buffered saline. After washing, polyps were stained with SYBR Gold (Life Technologies GmbH, Darmstadt, Germany) for 5 min. Before embedding in Mowiol/DABCO (Sigma-Aldrich, St Louis, MO, USA), animals were rinsed for 10 min in phosphate-buffered saline. Animals were analyzed using a Leica (Wetzlar, Germany) TCS SP5 confocal laser scanning microscope.

High-pressure freezing/freeze substitution fixation (HPF/FS) of Hydra glycocalyx

Hydra polyps immersed in Hydra culture medium were quickly dissected to fit into HPF specimen carriers. Tissue pieces are pipetted with Hydra culture medium into the cavity of a HPF aluminum platelet, which was 100 μm in depth and prefilled

with 1-hexadecene. This platelet was covered by a second one, inserted into a HPF specimen holder and high-pressure frozen using HPM 010 (Bal-Tec, Balzers, Liechtenstein). Before FS, the frozen 1-hexadecene was carefully removed under liquid nitrogen, and then the samples were transferred into precooled test tubes filled with acetone containing 1% (w/v) OsO_4 and 0.2% uranyl actetate. Dehydration was carried out at 90 °C in a conventional FS unit (AFS, Leica Microsystems, Vienna, Austria) for 24 h, followed by two further FS steps at 70 °C and 50 °C, each for 8 h. After FS, the temperature was raised up to 48 °C and samples were infiltrated at 48 °C with EPON according to the following protocol: specimens were (1) washed in pure acetone for 3×10 min, (2) infiltrated with 30% (v/v) EPON in acetone for 3 h, (3) then infiltrated with 70% EPON (v/v) in acetone for 3 h and (4) finally, three incubation steps in pure EPON, each for 2 h, were performed at room temperature. Ultrathin sections were counterstained with 2.5% uranyl acetate and lead citrate solution, and finally investigated in a Philips EM 208S transmission electron micrograph (Philips, Eindhoven, The Netherlands).

Chemical fixation of Hydra glycocalyx

Polyps were fixed in 2% paraformaldehyde, 2.5% glutaraldehyde, 75 mM L-lysine and 0.05% ruthenium red in 50 mM cacodylate buffer, pH 7.4, for 18 h at 4 °C. After washing with 75 mM cacodylate buffer for 30 min, postfixation was carried out with 1% OsO_4 and 0.05% ruthenium red in 75 mM cacodylate buffer for 2 h at 4 °C. After washing with 75 mM cacodylate buffer for 30 min, tissue was dehydrated in ethanol. For scanning electron microscopy, animals were critical point dried in an ethanol–carbon dioxide mixture (CPD030; Bal-Tec), sputter coated (SCD050; Bal-Tec) and viewed at 10 kV using S420 scanning electron microscope (LEO, Leica).

For transmission electron microscopy, animals were embedded in Agar 100 resin (Agar Scientific, Ltd, Stansted, UK). Ultrathin sections were contrasted with 2.5% uranyl acetate for 5 min and lead citrate solution (freshly prepared from lead acetate and sodium citrate) for 2 min and were analyzed using a Tecnai G^2 Spirit BioTWIN transmission electron microscope (FEI Company, Hillsboro, OR, USA).

Fluorescence in situ hybridization analysis of bacterial colonizers

Hydra polyps were washed in 500 µl phosphate-buffered saline for 2 min. The supernatant was transferred to a new tube and fixed by adding 500 µl 8% paraformaldehyde for 1 h. After fixation, supernatant was filtered through a white polycarbonate membrane filter (pore size: 0.2 µm). Afterwards, the filter was washed by 10 ml sterile H_20 and air-dried. Hybridizations of filters were done as described by Manz *et al.* (1992) with monofluorescently labeled ribosomal RNA (rRNA)-targeted oligonucleotide probes: positive control, universal eubacterial probe EUB338 5′-GCTGCCTCCCGTAG GAGT-3′, and negative control, EUB338 antisense probe non-EUB338 5′-ACTCCTACGGGAGGCAGC-3′. The phylotype-specific oligonucleotide probes (Fraune *et al.*, 2010) were designed using the computational tool Primrose 2.17 (Ashelford *et al.*, 2002). Probes were 5′ end-labeled with either Alexa Fluor 488 (Life Technologies GmbH) (green fluorescence) or Cy3 (Life Technologies GmbH) (red fluorescence). Hybridization was carried out at 46 °C for 90 min followed by one wash step at 48 °C for 15 min. The formamide concentration in the hybridization buffer varied between 0% and 30%, and the sodium chloride concentration in the post-hybridization buffer was adjusted accordingly. The fluorescence signal by all probes was stable; the intensity of the signals was stable between 0% and 20% formamide and decreased slightly at 30% formamide. With nontarget cells, there was no signal even under low-stringency conditions (no formamide). Therefore, we routinely used 10% formamide for single hybridizations and for double hybridizations with EUB338. In addition, samples were stained with Hoechst staining and mounted with Citifluor (Citifluor Ltd, London, UK). Examination was done at magnification of $\times 600$ with a Zeiss Axioskope 2 (Zeiss, Oberkochen, Germany).

Cultivation of Hydra-associated bacteria

Single Hydra polyps were placed in a 1.5-ml reaction tube and washed three times with 1 ml sterile filtered Hydra medium. After homogenization with a pestle, 100 µl (equates to 1/10 of a polyp) was plated on R2A agar plates (Sigma-Aldrich). After incubation at 18 °C for 5 days, single colony-forming units (CFUs) were isolated and cultivated in liquid R2A medium. The bacteria were identified by Sanger sequencing of the 16S rRNA gene and stocks were stored in Roti-Store cryo vials (Carl Roth, Karlsruhe, Germany) at -80 °C.

Isolation, culturing and identification of Fusarium sp.

Fungal hyphae were isolated from infected germ-free (GF) Hydra cultures and cultured on R2A agar plates at 18 °C for 3 days. Freshly grown hyphae were transferred to fresh agar plates or into a falcon tube containing 50 ml liquid R2A medium. For identification, fungal genomic DNA were extracted using polyps using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). The internal transcribed spacer (ITS) of the ribosomal nuclear DNA was amplified using the universal ITS1 and ITS4 primer pair, as described in Paul and Steciow (2004). The fungi ITS was sequenced by Sanger sequencing and compared with public database at NCBI (National Center for Biotechnology Information) using blast

searches. For phylogenetic analysis a sequence alignment for the ITS region was generated using MEGA5 (Tamura *et al.*, 2007). A model test was used to estimate the best-fit substitution models for phylogenetic analyses. For the maximum-likelihood analyses, genes were tested using the Kimura 2-parameter model + G model. A bootstrap test with 100 replicates for maximum likelihood and random seed was conducted.

Plate diffusion assay to test the in vitro activity of isolated bacteria against Fusarium sp.
Six isolated bacteria were tested alone or in combinations in a plate diffusion assay for their *in vitro* activity against the isolated *Fusarium* fungi. Therefore, 10 µl of a pure bacterial culture ($OD_{600} = 0.1$) or a mixture of two bacterial culture ($OD_{600} = 0.1$) was spotted into small holes (3 mm) on R2A agar plates. After 2 days of bacterial growth, 10 µl of fungal spores (~500 spores per µl) were added to the holes and fungal growth was quantified after 5 days by measuring the diameter of visible hyphae. Analyses of variance were used to test the effect of single bacterial isolates to fungal growth. Dunnett's test was used for a *post hoc* test to compare treatment with control samples. Two-way analysis of variance was used to test the interaction effect (synergy or antagonism) of two bacterial isolates to fungal growth.

Generation of GF Hydra
Polyps were incubated for 1 week in an antibiotic solution containing 50 µg ml^{-1} each of ampicillin, rifampicin, streptomycin and neomycin with daily exchange of the solution. After 1 week of treatment, the polyps were transferred into sterile-filtered and autoclaved Hydra medium and fed with GF *A. salina* larvae (hatched in 30‰ artificial sea water containing the same antibiotic solution). Following 1 week of recovery, the absence of bacteria was verified by plating homogenized polyps on R2A agar plates. After incubation at 18 °C for 5 days, the CFUs were counted. Absence of CFUs indicated successful antibiotic treatment.

For culture-independent analysis, total DNA was extracted from single polyps using the DNeasy Blood & Tissue Kit (Qiagen). The 16S rRNA genes were amplified using the universal primers Eub-27F and Eub-1492R (Weisburg *et al.*, 1991) in a 30-cycle PCR. Sterility was verified by the absence of a PCR product, whereas the positive control of none-treated polyps showed a signal.

Generation of mono- and di-associated Hydra
Bacteria isolated from Hydra polyps were cultured in liquid R2A medium for 3 days at 18 °C. Following centrifugation at 1380 × *g* for 10 min, the bacterial pellet was resuspended in sterile Hydra medium. Using a photometer, the optical density (OD_{600}) of each bacterial solution was adjusted to 0.1. For di-associations, both bacterial solution were mixed in a 1:1 ratio. GF Hydra polyps were incubated in these solutions for 24 h. Conventionalized polyps were incubated in a mixture of *Hydra vulgaris* (AEP) culture supernatant and *H. vulgaris* (AEP) tissue homogenates (one homogenated polyp per ml) instead. Nonassociated bacteria were removed by washing with sterile Hydra medium after 24 h. Following another 24 h, the successful re-association was checked by plating tissue homogenates on R2A agar plates and counting CFU/polyps. Statistical analysis of the bacterial load was conducted using analysis of variance. Dunnett's test was used as a *post hoc* test to compare treatment with control samples.

In vivo infection experiments with Fusarium sp.
The fungi *Fusarium sp.* was cultured on R2A agar plates. A piece of hyphae containing agar was transferred into a falcon tube, containing 50 ml liquid R2A medium. The tube was sealed and incubated at room temperature for 48 h. Fungal spores were retrieved from the supernatant and transferred into 1.5 ml reaction tubes. After centrifugation at 20 000 × *g* for 5 min, the pellet was resuspended in 1/10 of the original volume using sterile Hydra medium. For fungal infection, groups of five *Hydra* polyps were placed in a volume of 480 µl sterile Hydra medium using 1.5 ml tubes. Each treatment was repeated between 18 to 44 times (see Figure 5c). All re-associated *Hydra* polyps were infected with 20 µl spore solution (~500 spores per µl) from the supernatant of a 48-h-old fungal culture. Fungal growth was monitored 7 days post infection by the outgrowth of hyphae. If fungal hyphae were detectable around the polyps, the tube was counted as 'infected'. In case of no detectable hyphae, the tube was counted as 'uninfected'. Statistical analyses were conducted by Fisher's exact test to test whether bacterial recolonization of polyps caused different infection rates compared with GF or control polyps.

To test whether bacterial di-associations possess synergistic or antagonistic activities, we used a generalized linear model (function glm() from stats package in R), with individual infection as response. The different bacteria and all experimentally tested interactions were used as explanatory factors. We performed model selection using the drop1() function from the stats package. The best model was selected based on Akaike's information criterion, a measure for the relative quality of a model. We used analysis of deviance for significance testing of the remaining factors within the chosen model (the model with the lowest Akaike's information criterion). All significant interaction terms indicate synergistic or antagonistic effects of bacterial colonizers.

Results

The Hydra ectoderm is covered by a multilayered glycocalyx that is a habitat for a complex bacterial community

Using HPF/FS we first confirmed earlier observations (Holstein *et al.*, 2010; Böttger *et al.*, 2012) that the *Hydra* ectoderm is covered by a multilayered glycocalyx. Transmission electron microscopy revealed five distinct layers (c1–5) in the glycocalyx that together extend up to 1.5 µm from the cell surface (Figure 1b). The c1 layer is closely associated with the ectodermal cells, whereas the layer

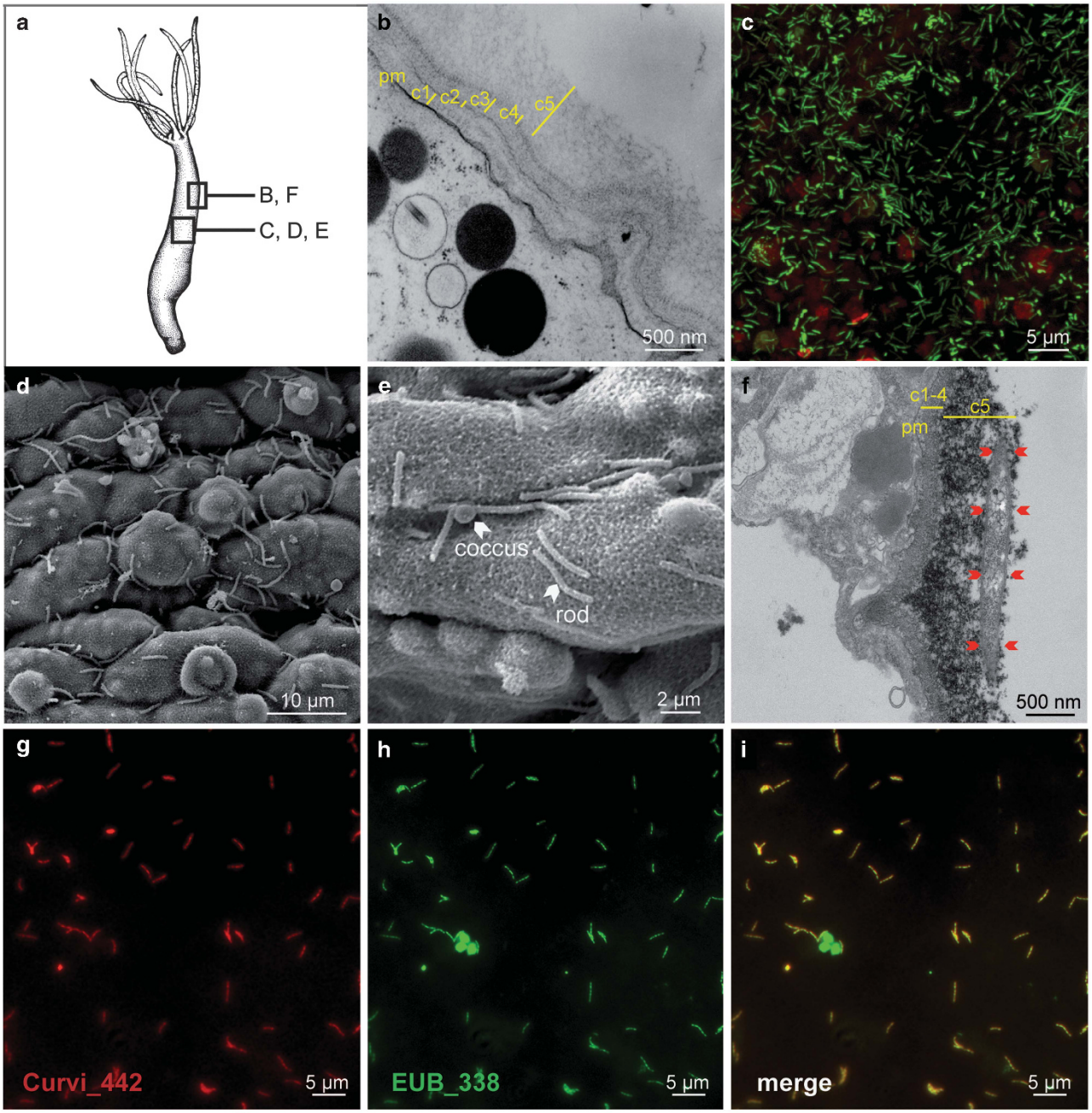

Figure 1 *Hydra* ectodermal glycocalyx is colonized by a complex bacterial community. (**a**) Schematic drawing of the freshwater polyp *Hydra* indicating the tissue areas in which the glycocalyx and the bacterial colonization was examined. The letters correspond to further panels in this figure. (**b**) *Hydra* ectodermal epithelial cells prepared by HPF/FS fixation provide excellent preservation of the glycocalyx layer revealing five distinct layers (c1–c5); pm, plasma membrane. (**c**) Total bacterial community colonizing the surface of the ectodermal epithelium in *Hydra*, stained with SYBR gold. (**d**, **e**) Raster electron micrograph (REM) of bacterial cells located on the surface of ectodermal cells. (**f**) Transmission electron micrograph (TEM) of a rod-shaped bacterium (red arrows) located within the outer layer (c5) of the glycocalyx covering ectodermal epithelial cells. (**g–i**) Fluorescence in situ hybridization (FISH) analysis of bacteria removed from the ectodermal epithelium. Bacteria cells were stained with the phylotype-specific probe for *Curvibacter sp.* (Curvi_442) (**g**) and with the eubacterial oligonucleotide probe EUB338 (**h**). Overlay images indicating the specifically labeled bacteria in yellow (**i**).

c5 is made by a loose meshwork accounting for >50% of the glycocalyx. Numerous electron-dense vesicles within the ectodermal epithelial cell (Figure 1b) indicate that glycocalyx components get secreted by ectodermal epithelial cells.

To localize the commensal microbiota, we initially used SYBR Gold staining. Epifluorescence microscopy uncovered (Figure 1c) a dense and morphologically heterogeneous community of bacteria colonizing the ectodermal epidermis. We next used scanning electron microscope investigations of chemical-fixed *Hydra* to better visualize the external appearance of the bacteria. In all specimens examined, rod-shaped as well as cocci bacteria were found attached to the ectodermal epithelial cells (Figures 1d and e), indicating that *Hydra* hosts a morphologically diverse microbial community. Next, we wanted to address whether bacterial colonizers live within the glycocalyx and if so where precisely. Transmission electron microscopy localized the commensal bacteria in the loose outer layer (c5) of the glycocalyx (Figure 1f), whereas the inner attached layers c1–4 were never observed to contain bacteria. This indicates that *Hydra* inner glycocalyx layers closely associated with the ectodermal epithelial cells are impenetrable to bacteria and may function as a protective barrier for the epithelial cell surface. The dominant member of the bacterial community colonizing *H. vulgaris* (AEP) tissue is *Curvibacter spec.* (Franzenburg *et al.*, 2013b): *Curvibacter sp.* was co-sequenced with the *Hydra magnipapillata* genome (Chapman *et al.*, 2010) and dominates 16S rRNA gene libraries in *H. vulgaris* (AEP) and *H. magnipapillata* (Franzenburg *et al.*, 2013a, b). To analyze whether *Curvibacter* is living within the glycocalyx, polyps were washed sequentially with high salts as previous observations in our laboratory indicated that the glycocalyx is shed under hypertonic conditions. The supernatant was subsequently fixed onto membrane surfaces and subjected to fluorescence *in situ* hybridization with phylotype-specific probes for *Curvibacter sp.* (Curvi442) (Fraune *et al.*, 2010). This assay demonstrated (Figures 1g–i) that *Curvibacter* is a rod-shaped bacterium that is localized in the glycocalyx at the surface of the epithelium.

GF polyps are prone to infection by the filamentous fungus Fusarium sp.

Although the above observations suggest that *Hydra* surface is densely colonized by a distinct bacterial community, the role of the commensal bacteria that thrive on the ectodermal epithelium remained unclear. In order to address microbial functions in pathogen defense, we analyzed the outcome of fungal infection in GF *Hydra*. Although control *H. vulgaris* (AEP) cultures normally do not show any signs of fungal infection (Figure 2a), GF *H. vulgaris* (AEP) cultures are often infected by fungi (Figures 2b and c). Fungal hyphae are growing on the surface of GF polyps closely attached to the ectodermal epithelium-producing spores that subsequently can get released to the surrounding water (Figure 2c). In line with the hypothesis that members of the normal microbiota residing on the surface of the polyps may play a key role in pathogen defense, untreated fungal infections of GF polyps frequently cause the death of the animals.

Using standard culturing conditions we isolated fungal hyphae from infected *Hydra* polyps to establish a pure fungal culture growing both on plates and in liquid medium (Figures 2d and e). Sequencing of the ITS (ITS1 and ITS2) ribosomal DNA identified the pathogenic fungi as *Fusarium sp.* (also known as *Gibberella sp.*) (Figure 2f), a filamentous fungus belonging to the order Hypocreales.

In vitro antifungal activity of single bacterial isolates

To dissect the pathogen defense potential of individual members of *Hydra* complex microbiota, we isolated and cultured six different bacterial strains from *H. vulgaris* (AEP) epithelium. In order to verify their host specificity, we analyzed their presence in 16S rRNA libraries (Franzenburg *et al.*, 2013b). All six cultivated bacteria could be confirmed independently by a culture-independent method (454 pyrosequencing of 16S rRNA genes) to be present in the bacterial community of *H. vulgaris* (AEP) (Table 1). Whereas five bacterial strains belong to the Burkholderiales within the Betaproteobacteria, one bacterial strain belongs to the Pseudomonadales within the Gammaproteobacteria. These six cultivated bacteria represent 90.0 ± 2.3% of the bacterial abundance in *H. vulgaris* (AEP) (Table 1) characterized previously (Franzenburg *et al.*, 2013b). Therefore, these six cultivated bacteria are good representatives for the bacterial composition of *H. vulgaris* (AEP) that is dominated by Betaproteobacteria of the order Burkholderiales.

To monitor their impact on fungus growth, all six bacterial strains were investigated in an *in vitro* assay for the ability to prevent *Fusarium sp.* germination and mycelia growth. The activities of the isolated bacterial strains against the pathogenic fungus were examined by a dual-culture plate method. Interestingly, in this *in vitro* assay the majority of resident bacteria including the main colonizer *Curvibacter* showed only a minor or no ability to inhibit *Fusarium sp.* outgrowth after 5 days of incubation (Figure 3). Only one bacterial strain, *Pelomonas sp.*, exhibit strong inhibitory activity *in vitro* against the pathogenic fungus.

Bacteria–bacteria interactions increase antifungal activity in vitro

To assess the possibility that bacteria–bacteria interactions facilitate the observed antifungal

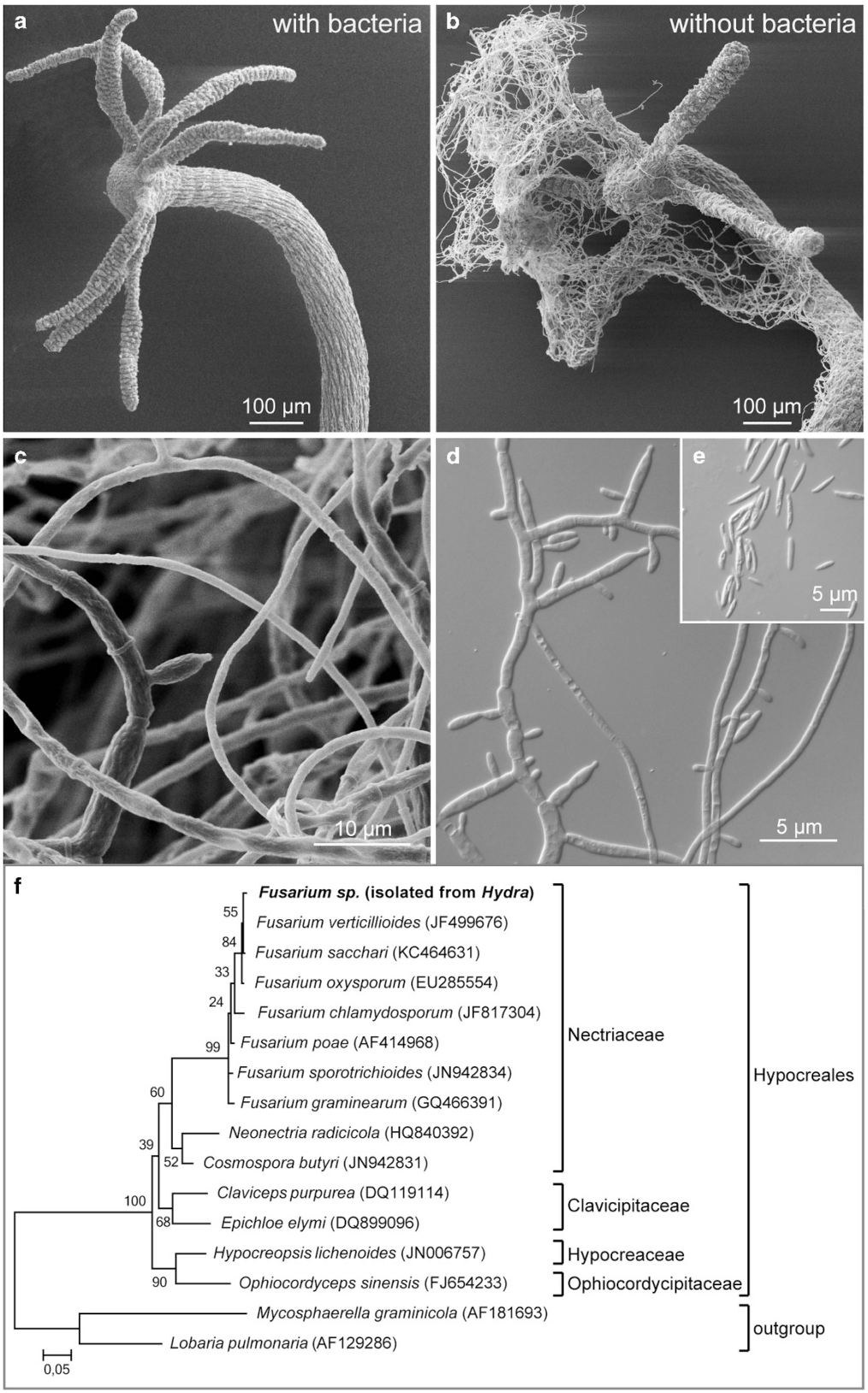

Figure 2 GF *Hydra* polyps are prone to fungal infection by *Fusarium sp.* (**a**) Raster electron micrograph (REM) of a control polyp showing no fungal infection. (**b**) GF *Hydra* polyp infected *by Fusarium sp.* (**c**) Fungal hyphae in association with *Hydra* producing a spore. (**d**) Fungal hyphae grown in liquid R2A medium. (**e**) Spores isolated from the supernatant of a liquid *Fusarium sp.* culture. (**f**) Phylogenetic position of *Fusarium sp.* (isolated from infected *Hydra*) within the Nectriaceae (based on ITS region, maximum likelihood using Kimura 2-parameter model + G). Bootstrap values are shown at the corresponding nodes. The branch-length indicator displays 0.05 substitutions per site.

Table 1 Bacterial strains cultivated from *Hydra vulgaris* (AEP)

Bacterium	*Consensus lineage*	*Clone*	*CFUs per ml*[a]	*Relative abundance (%)*[b]		*OTU ID*[c]	e-*Value*[d]	*Acc. no.*[e]
				Mean (n = 3)	*s.d.*			
Curvibacter sp.	Betaproteobacteria; Burkholderiales; Comamonadaceae	AEP1.3	2×10^8	75.6	7.9	233	e − 152	KJ187967
Undibacterium sp.	Betaproteobacteria; Burkholderiales; Oxalobacteraceae	C1.1	7×10^8	2.1	2.6	60	0.0	KJ187965
Duganella sp.	Betaproteobacteria; Burkholderiales; Oxalobacteraceae	C1.2	5×10^7	11.1	5.7	245	e − 180	KJ187966
Acidovorax sp.	Betaproteobacteria; Burkholderiales; Comamonadaceae	AEP1.4	5×10^7	0.7	0.3	49	e − 153	KJ187968
Pelomonas sp.	Betaproteobacteria; Burkholderiales; Comamonadaceae	AEP2.2	1×10^8	0.2	0.1	14	e − 136	KJ187969
Pseudomonas sp.	Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae	C2.2	3×10^8	0.4	0.4	282	e − 134	KJ187970
				Σ90.1				

Abbreviations: Acc. no., accession number; CFU, colony-forming unit; OTU, operational taxonomic unit.
[a]CFUs per ml at $OD_{600\,nm} = 0.1$.
[b]Relative abundances were calculated based on three previously published (Franzenburg *et al*., 2013b) 16S rRNA gene libraries of *Hydra vulgaris* (AEP) sequenced by 454 pyrosequencing.
[c]OTU number according to sequence libraries obtained from *Hydra vulgaris* (AEP) (Franzenburg *et al*., 2013b) using 454 pyrosequencing.
[d]The *e*-values were determined by blastn algorithm comparing 16S rRNA genes from bacterial isolates with sequence libraries obtained from *Hydra vulgaris* (AEP) (Franzenburg *et al*., 2013b) using 454 pyrosequencing.
[e]Sequences have been deposited in GenBank.

resistance of control polyps, we tested pair-wise combinations of all six isolated bacteria *in vitro*. In comparing the antifungal activity of bacterial isolates alone with the activity of the pair-wise cultured bacteria, we were able to show that most of the co-cultures show a greater antifungal activity than the corresponding bacteria alone (Table 2). Two-way analysis of variance suggests that in most combinations the bacteria act in an additive manner against *Fusarium sp.* (Table 2 and Figure 4a). Interestingly, one bacterial combination (*Undibacterium sp./Acidovorax sp.*) acts synergistically to inhibit the fungal growth *in vitro* (Table 2 and Figure 4b). In contrast, two bacterial co-cultures (*Pelomonas sp./Undibacterium sp.* and *Pelomonas sp./Duganella sp.*) act in an antagonistic manner (Table 2 and Figure 4c). In both cases the strong antifungal activity of *Pelomonas sp.* alone is reduced in combination with each of the two other bacteria.

Bacteria–bacteria interactions are also needed in vivo to provide full protection

We wanted to address the *in vivo* relevance of the *in vitro* results and the importance of bacteria–bacteria interaction for the antifungal activity in the native *Hydra* host. To uncover this, we established a gnotobiotic Hydra model that was selectively colonized with one or two of the six bacterial strains (Figure 5a).

For di-associations, we always used *Curvibacter sp.* in combination with one of the five other bacterial isolates, as *Curvibacter sp.* is the most dominant colonizer (~75%, see Table 1) in the natural bacterial community of *H. vulgaris* (AEP).

As controls we tested wild-type, conventionalized (that is, ex GF polyps re-infected with a complex microbiota) and GF polyps. To evaluate the effectivity of recolonization, we first monitored the bacterial load by assessing the bacterial CFUs per polyp (Figure 5b). In mono-association, only *Acidovorax sp.* showed increased bacterial load compared with control polyps. All other mono-associations resulted in bacterial loads comparable to control polyps, indicating that available niches can be colonized by all bacteria tested (Figure 5b). Similarly, only di-association with *Curvibacter sp.* and *Acidovorax sp.* yielded higher bacterial loads when compared with control polyps. All other di-associations showed no differences in bacterial load compared with control polyps (Figure 5b).

To examine antifungal activity in *Hydra* that were selectively colonized with one or two of the six bacterial strains, polyps were screened for the presence or absence of fungal hyphae 7 days post infection with 20 µl spore solution (~500 spores per µl). To test for differences in infection rates between controls (GF and control) and recolonized polyps, we used Fisher's exact test. We compared all treatments with GF and control polyps, respectively (Figure 5c). As shown in Figure 5d, GF polyps were highly susceptible to fungal outgrowth, whereas control polyps largely inhibited fungal growth. The re-introduced complex microbiota (conventionalized) provided the same resistance against fungal infection as observed in control polyps, indicating that the resident microbiota facilitates fungal

clearance and also that the antibiotic treatment *per se* does not lead to host tissue damage to foster *Fusarium* outgrowth (Figure 5c).

Individual effects of single bacterial isolates were significant for *Curvibacter sp.*, *Undibacterium sp.*, *Acidovorax sp.* and *Pelomonas sp* compared with GF polyps (Figure 5c). Strikingly, none of the mono-associated polyps provided the polyps with the same rate of resistance as control or conventionalized polyps. Surprisingly, *Pelomonas sp.*, which inhibits fungal growth significantly *in vitro* (Figure 3), showed no antifungal activity *in vivo* (Figure 5c). Vice versa, *Acidovorax sp.*, which showed only weak effect *in vitro*, appears to have strong antifungal activity *in vivo.*

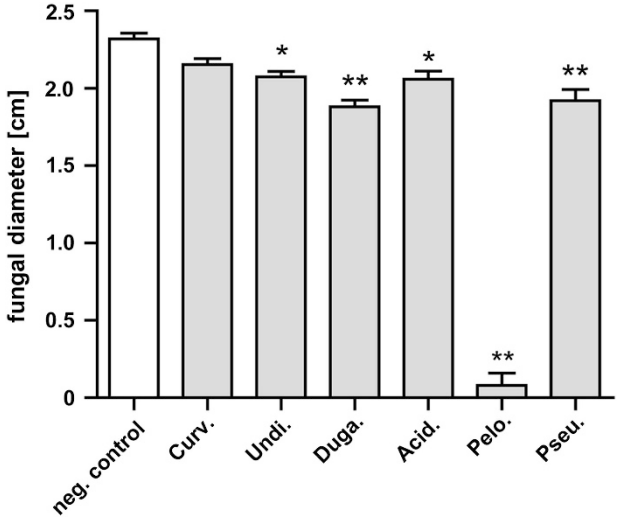

Figure 3 *In vitro* activity of bacterial isolates against *Fusarium sp. In vitro* plate diffusion assay for fungal inhibition by bacteria isolated from *Hydra* tissue. Statistical analysis was conducted using analysis of variance (ANOVA; $*P<0.05$, $**P<0.01$, $***P>0.001$; $n=5$. Acid., *Acidovorax sp.*; Curv., *Curvibacter sp.*; Duga., *Duganella sp.*; Pelo., *Pelomonas sp.*; Pseu., *Pseudomonas sp.*; Undi., *Undibacterium sp.*).

Within the di-associations, three combinations possess a significant activity against fungal infection compared with GF polyps (Figure 5c). Interestingly, two combinations (*Curvibacter sp./Duganella sp.* and *Curvibacter sp./Pelomonas sp.*) are as active as control polyps against fungal infections. In contrast, two combinations (*Curvibacter sp./Acidovorax sp.* and *Curvibacter sp./Pseudomonas sp.*) exhibit no antifungal activity (Figure 5c).

To evaluate the contribution of bacteria–bacteria interaction to the observed antifungal activity in di-associations in more detail, we used a generalized linear model (Table 3). We found three significant bacteria–bacteria interactions contributing to fungal infection rates in di-associations (Table 3). Whereas two interactions, *Curvibacter sp./Undibacterium sp.* and *Curvibacter sp./Acidovorax sp.*, exhibit an antagonistic effect on fungal infection, the combination of *Curvibacter sp./Duganella sp.* exhibit a synergistic effect (Table 3). The strong reduction of infection rate of the combination of *Curvibacter sp./Pelomoans sp.* can be explained by an additive effect of the individual effects of both bacteria.

Discussion

In this study, we have examined the localization and pathogenic fungus clearance potential of members

Table 2 *In vitro* bacterial activities against *Fusarium sp.*

Bacteria 1			*Fungal growth*[a]			P-*values*			
	Bacteria 2	N	*Bacteria 1* ± *s.d.*	*Bacteria 2* ± *s.d.*	*Co-culture* ± *s.d.*	*Bacteria 1*	*Bacteria 2*	*Interaction*[b]	*Biological effect*
Curvibacter sp.	*Undibacterium sp.*	9	0.94 ± 0.07	0.84 ± 0.20	0.84 ± 0.11	0.3684	0.0002	0.2327	Additive
Curvibacter sp.	*Acidovorax sp.*	9	0.99 ± 0.05	0.86 ± 0.14	0.77 ± 0.13	0.1151	<0.0001	0.2044	Additive
Curvibacter sp.	*Pelomonas sp.*	9	0.94 ± 0.07	0.02 ± 0.03	0.01 ± 0.03	0.0466	<0.0001	0.1491	Additive
Curvibacter sp.	*Duganella sp.*	9	0.93 ± 0.06	0.94 ± 0.09	0.86 ± 0.08	0.0028	0.0081	0.9174	Additive
Curvibacter sp.	*Pseudomonas sp.*	6	0.93 ± 0.09	0.85 ± 0.11	0.79 ± 0.07	0.0631	0.0002	0.7320	Additive
Undibacterium sp.	*Acidovorax sp.*	9	0.95 ± 0.14	0.99 ± 0.14	0.77 ± 0.22	0.0019	0.0143	0.0477	Synergistic
Undibacterium sp.	*Pelomonas sp.*	5	0.76 ± 0.13	0.01 ± 0.02	0.60 ± 0.11	0.0004	<0.0001	<0.0001	Antagonistic
Undibacterium sp.	*Duganella sp.*	9	0.84 ± 0.17	0.88 ± 0.14	0.77 ± 0.08	0.0002	0.0071	0.3451	Additive
Undibacterium sp.	*Pseudomonas sp.*	6	0.82 ± 0.18	0.83 ± 0.09	0.74 ± 0.11	0.0022	0.0078	0.2808	Additive
Duganella sp.	*Acidovorax sp.*	9	0.93 ± 0.13	0.90 ± 0.18	0.81 ± 0.11	0.0342	0.0035	0.8419	Additive
Duganella sp.	*Pelomonas sp.*	9	0.84 ± 0.14	0.02 ± 0.03	0.90 ± 0.12	<0.0001	<0.0001	<0.0001	Antagonistic
Duganella sp.	*Pseudomonas sp.*	6	0.92 ± 0.11	0.88 ± 0.05	0.80 ± 0.08	0.0109	0.0004	1.0000	Additive
Acidovorax sp.	*Pelomonas sp.*	6	1.00 ± 0.03	0.04 ± 0.05	0.02 ± 0.03	0.8504	<0.0001	0.8504	Additive
Acidovorax sp.	*Pseudomonas sp.*	6	0.88 ± 0.13	0.79 ± 0.07	0.73 ± 0.09	0.0063	<0.0001	0.4379	Additive
Pelomonas sp.	*Pseudomonas sp.*	6	0.03 ± 0.04	0.81 ± 0.08	0.02 ± 0.02	<0.0001	0.0005	0.0057	Additive

Two-way analysis of variance (ANOVA) was used to determine whether bacterial isolates exhibit in combinations an additive, a synergistic or an antagonistic inhibition of *Fusarium sp.*

[a]Fungal growths were normalized in each individual experiment to its own control (100%).

[b]Interaction *P*-values of <0.05 indicate synergistic or antagonistic interactions of the co-cultured bacteria; *P*-values of >0.05 indicate additive activities of co-cultured bacteria.

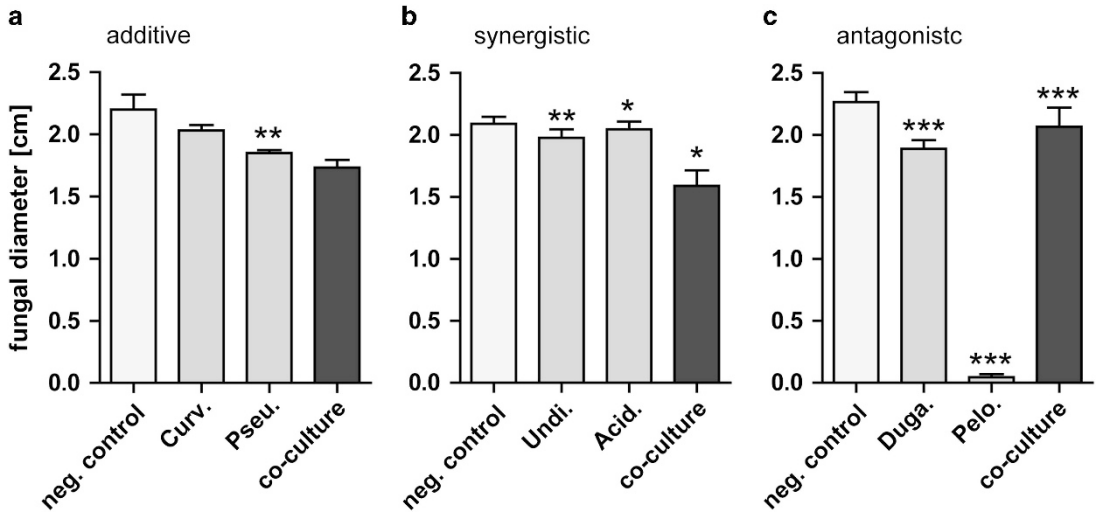

Figure 4 Examples of *in vitro* activity of co-cultured bacteria against *Fusarium sp.* (**a**) Example of an additive effect of two bacterial isolates in a plate diffusion assay. (**b**) Example of a synergistic effect of two bacterial isolates. (**c**) Example of an antagonistic effect of two bacterial isolates. Statistical analysis was conducted using two-way analysis of variance (ANOVA) to test the interaction effect (synergy or antagonism) of two bacterial isolates to fungal growth (*$P<0.05$, **$P<0.01$, ***$P>0.001$) (see also Table 2). Acid., *Acidovorax sp.*; Curv., *Curvibacter sp.*; Duga., *Duganella sp.*; Pelo., *Pelomonas sp.*; Pseu., *Pseudomonas sp.*; Undi., *Undibacterium sp.*

of *H. vulgaris* (AEP) resident microbiota. We found that the bacterial colonizers in *Hydra* inhabit the outer layer of the glycocalyx and, therefore, appear to have no direct contact to the ectodermal epithelium (Figure 1f). Thus, the glycocalyx seems on one hand to separate the bacterial cells from the epithelium and on the other hand to provide a habitat for the bacterial colonizers. This principle of separation into a habitat for symbiotic bacteria and a physical barrier preventing excessive immune activation was previously described for the mucosal surface of the mammalian colon, where a mucous layer is restricting bacterial colonizers to the outer loose mucus layer whereas the inner mucus layer is devoid of bacteria (Johansson *et al.*, 2008, 2011). As such a glycoprotein-covered barrier epithelium can be traced back to the ancestral metazoan *Hydra*, it apparently is a conserved feature shared by many multicellular animals.

We also discovered that *Hydra* polyps, when artificially deprived of their specific bacterial colonizers, are prone to fungal infection by the filamentous fungus *Fusarium sp.* Spores of *Fusarium sp.* seem to be continuously present in the laboratory environment surrounding the *Hydra* polyp. Our observations indicate that the specific microbiota (Fraune and Bosch, 2007; Franzenburg *et al.*, 2013b) colonizing the interface between *Hydra* host ectodermal epithelium and the environment provide efficient protection against fungal infection.

We identified several bacterial colonizers, including *Acidovorax sp.*, *Curvibacter sp.*, *Pelomonas sp.* and *Undibacterium sp.*, that significantly inhibit fungal outgrowth *in vivo* (Figure 5c). None of these bacteria were previously reported to synthesize antifungal compounds, although *Acidovorax sp.* and *Curvibacter sp.* are reported as symbionts in other organisms (Schramm *et al.*, 2003; McKenzie *et al.*, 2012). Most importantly, we have observed that none of the tested bacterial colonizers alone was able to provide full antifungal resistance (Figure 5c). In contrast, resistance, observed in control polyps, was achieved in polyps recolonized by a complex bacterial community (conventionalized) indicating that bacteria–bacteria interactions contribute to the full resistance against fungal infections. Our *in vivo* and *in vitro* results indicate that bacterial colonizers of *H. vulgaris* (AEP) interact in a complex manner and that the sum of additive, synergistic as well as antagonistic effects may gives rise to the overall resistance of the holobiont *Hydra* against fungal infections. Interestingly, the two most dominant bacterial colonizers *Curvibacter sp.* and *Duganella sp.* exhibit weak or no activity alone, but exhibit a strong synergistic effect in di-association, reducing the rate of infected polyps to 15%. This fact points to the *in vivo* importance of these two main colonizers for fungal clearance. In sum, this study provides first experimental evidence for the view that in animals at the base of metazoan evolution a complex microbiota is necessary and sufficient for pathogen clearance. The study also demonstrates that mono-associated bacteria in most cases fail to function efficiently in pathogen defense.

The observations in *Hydra* are in line with studies in the locust *Schistocerca gregaria* where species-rich bacterial communities provide better protection against pathogen invasion than species-poor communities (Dillon *et al.*, 2005). The findings make it likely that an 'unfavorable' microbiota composition or fluctuating bacterial community composition may result in disturbed immune function of the

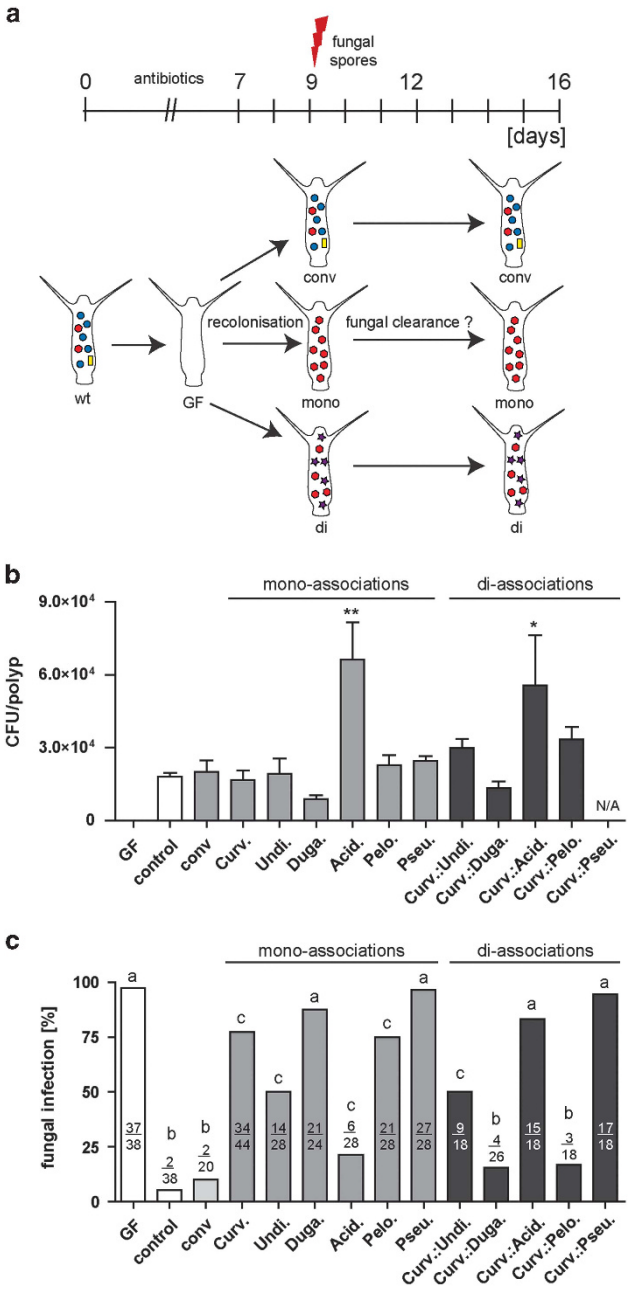

Figure 5 *In vivo* infection rates of *Hydra* polyps recolonized by different bacterial isolates. (**a**) Experimental set-up for mono- and di-associated and conventionalized (conv) *Hydra* polyps used for fungal infection experiments. (**b**) Bacterial load of recolonized *Hydra* polyps. N/A indicates 'not available' as *Pseudomonas sp.* shows swarming behavior and thereby overgrew *Curvibacter sp.*, $n \geq 4$. (**c**) *In vivo* infection rates with *Fusarium sp.* after inoculation with spores. Statistical analyses were conducted by Fisher's exact test. Different lowercase letters indicate significant differences between treatments: 'a' indicates significantly different from control ($P<0.01$), 'b' indicates significantly different from GF ($P<0.01$), 'c' indicates significantly different from control and GF ($P<0.01$). Fraction numbers indicate x infected cases per n replicates. Acid., *Acidovorax sp.*; Curv., *Curvibacter sp.*; Duga., *Duganella sp.*; Pelo., *Pelomonas sp.*; Pseu., *Pseudomonas sp.*; Undi., *Undibacterium sp.*

Table 3 Results of a generalized linear model (GLM) of *Fusarium sp.* infection rates

Factor	*LR ChiSq*	*d.f.*	*Pr (>ChiSq)*
Curv.	23.868	1	1.03e−06***
Undi.	39.334	1	3.57e−10***
Acid.	75.699	1	<2.2e−16***
Pelo.	36.308	1	1.69e−09***
Curv./Undi.	8.654	1	0.003264**
Curv./Duga.	38.513	2	4.34e−09***
Curv./Acid.	39.693	1	2.97e−10***

Abbreviations: Acid., *Acidovorax sp.*; ChiSq, χ^2; Curv., *Curvibacter sp.*; Duga., *Duganella sp.*; LR, likelihood ratio; Pelo., *Pelomonas sp.*; Undi., *Undibacterium sp.*
Statistical analysis was conducted by GLM, with individual infection as response. Levels of significance of GLM model fits were tested using analysis of deviance with χ^2 distribution (**$P<0.01$ and ***$P>0.001$). Pr values of <0.05 for mono-associations indicate significant differences to GF polyps and Pr values of <0.05 for di-associations indicate significant interactions of these combinations, indicating synergistic (Curv./Duga.) or antagonistic (Curv./Undi. and Curv./Acid) interactions.

whole metaorganism. To ensure continuous protection by specific bacteria, host mechanisms controlling bacterial colonization are required. In *Hydra*, we have shown that the expression of species-specific antimicrobial peptides are key factors in maintaining a species-specific bacterial colonization (Fraune *et al.*, 2010; Franzenburg *et al.*, 2013b). In addition, active immune signaling via the Toll-like receptor cascade is involved in the re-establishment of bacterial homeostasis following disturbance (Franzenburg *et al.*, 2012) and, therefore, enhances the resilience of the bacterial community in *Hydra*.

Interestingly, the *in vivo* antifungal activity did not match the results obtained from *in vitro* experiments. To explain this discrepancy we offer four possible scenarios. First, certain Hydra-associated bacteria induce the production of host-derived antifungal compounds in *Hydra*. Second, the bacterial population density and the ratio of both bacteria in co-culture may differ between *in vitro* and *in vivo* experiments. As it was not possible to estimate the ratio of bacteria in co-culture as most tested bacteria morphologically do not differ significantly on agar plates, quantitative real-time PCR assays for unequivocal identification of the bacteria are under investigation. Third, the bacterial symbionts produce the antifungal compound only in association with the *Hydra* tissue, likely altering their metabolic state when changing their lifestyle from a free-living state to an epithelium colonizer. Fourth, antifungal compounds produced by the host and by the bacterial symbionts act together to inhibit fungal growth (Myers *et al.*, 2012). Collectively, the observed differences between the *in vitro* and *in vivo* data suggest that simplified measures of *in vitro* microbial function may be insufficient or even misleading for evaluating the pathogen clearance potential of resident microbes.

How does the microbiota efficiently prevent growth of pathogenic fungi? The contributions of

specific bacteria-derived molecules to immune defense against fungal pathogens are just beginning to be deciphered. Observations in a number of animal models provide hints that many associated symbionts serve a direct protective function for their host against fungal infections by producing antifungal substances. 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 (Gil-Turnes *et al.*, 1989). A different example is the infectious disease chytridiomycosis, caused by the fungal pathogen *Batrchochytrium dendrobatis*, that is a major factor responsible for the worldwide decline of amphibian species (Skerratt *et al.*, 2007). In this well-studied case, commensal bacteria have been shown to inhibit the growth of *B. dendrobatis* by the production of antifungal molecules like indole-3-carboxaldehyde or violacein (Brucker *et al.*, 2008; Harris *et al.*, 2009). Susceptibility to *B. dendrobatis* infection varies among amphibian species, and even within species some populations can coexist with *B. dendrobatis* whereas others decline to extinction. These differences in disease susceptibility have been correlated with the diversity of antifungal bacteria associated with a given frog population (Woodhams *et al.*, 2007). Interestingly, *Curvibacter* species are also associated with a variety of amphibian species (McKenzie *et al.*, 2012; Loudon *et al.*, 2013), but were not yet shown to produce antifungal compounds. Another prominent example for fungal defense by symbiotic bacteria is present in fungus-growing ants. These ants grow fungal cultivars for their nutrition that are prone to infection by the parasitic fungus *Escovopsis sp*. To defend their fungal cultivar against *Escovopsis sp*., leaf-cutter ants use symbiotic actinobacteria of the genus *Pseudonochardia* that are housed in specialized cuticular structure on the ant's body (Caldera *et al.*, 2009). These symbiotic bacteria produce the cyclic depsipeptide dentigerumycin that acts highly specific against *Escovopsis sp.*, without harming the fungal cultivar (Oh *et al.*, 2009). Thus, symbiotic bacteria are an integral part of antifungal immunity in a variety of organisms, offering an opportunity to resist fungal infection by a spread of bacterial symbionts.

The observations also support the view that because of bacterial colonizers *Hydra* might be able to adapt to new environmental conditions much faster than by genomic recombination. Thus, the microbiota is a complex trait that is under strong host genetic control. The resilience of complex and specific bacterial communities may be a critical factor to host health.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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