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MicroMeeting

Immunity and symbiosis

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Summary

The invertebrate immune system, which has become a major research focus, shares basic features of innate immunity with vertebrates and men. A special feature apparently found only in invertebrates is their close association with vertically heritable symbiotic microorganisms. The validity of the simple view of symbiosis as a mutually beneficial interaction between two uneven partners mainly improving the nutritional state of the two companions has been challenged, however, as symbiotic interactions might involve more partners, and symbiotic functions of the microorganisms are much more diverse than previously assumed. Likewise, microorganisms considered to be mostly harmful to their hosts have been shown to enhance host fitness under some circumstances. The role of a symbiont itself might change between environments or life stages of the host and symbionts might have features previously thought to be specific for pathogens. Understanding symbiotic interactions requires the comprehension of the cross-talk between the symbiotic companions, and the dissection of how long-lasting infections are established without eliminating the symbiont by host immune responses. Fascinating new findings in this field revealed that symbiosis might contribute to defence against pathogens or natural enemies. New symbiont-based approaches to defeat agricultural pests or pathogen transmission by arthropod vectors are becoming conceivable.

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Introduction

Invertebrates constitute more than 95% of all animal life on earth. Despite the enormous diversity of these animals, very little is known about their defence mechanisms against pathogenic microorganisms and parasites beyond major model systems. One major difference to vertebrates is that invertebrates appear to rely exclusively on innate immune responses, as no classical adaptive immune system is present (Ferrandon *et al.*, 2007; Vallet-Gely *et al.*, 2008). This innate immune system is able to discriminate between self and non-self and has some specificity, as it is able to distinguish between different types of microbe by the use of so-called pattern recognition receptors (PRRs) recognizing different microorganism-associated molecular patterns (MAMPs). The best-investigated innate immune system of invertebrates is that of *Drosophila melanogaster*. Briefly, distinguishing features of Gram-positive and Gram-negative bacteria are their different types of peptidoglycan, containing lysine in most Gram-positive bacteria and diaminopimelic acid in Gram-negative bacteria. Fungi are generally recognized by characteristic β-glucans. The various PRRs feed into different signal transduction pathways, mainly the Toll-pathway and the Imd-pathway, which activate NF-κB-like transcription factors and induce production of antimicrobial effectors including antimicrobial peptides (AMPs) that are active against the different types of microbes. This humoral arm of the innate immune system is complemented by the cellular arm, which mainly involves the action of phagocytic cells called haemocytes that circulate in the haemolymph or reside in different

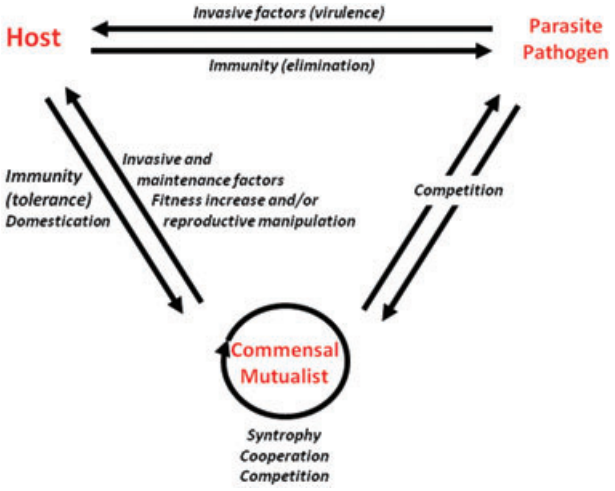

Fig. 1. An emerging general scheme showing possible relationships between hosts, pathogens and commensals/mutualists with each other.

tissues. Larger aggressors, such as parasitic wasps that lay eggs in the animal body cavities, can be defeated by encapsulation and melanization of the egg, mediated through a combination of cellular systems, and induction of the phenoloxidase cascade respectively.

A further striking difference between invertebrates and vertebrates is that many invertebrates have evolved intimate associations with symbiotic and frequently beneficial microorganisms (Buchner, 1965). Many such symbiotic microorganisms can be found intracellularly, frequently in specialized cells called bacteriocytes that are provided by the animals. Others are found extracellularly in special compartments provided by the host, such as gut crypts (Baumann, 2005; Kikuchi *et al*., 2009), with a minority found extracellularly within the hemocoel of the host. The manifold advantages of such interactions for the animals range from nutritional contribution to mediation of pathogen and parasite defence. Figure 1 shows an emerging scheme with possible relationships between symbionts/mutualists, pathogens and their hosts. In many cases, the symbiotic interactions are obligate for the 'host' partner as well as for the symbiont, but a rapidly increasing number of facultative secondary endosymbioses have been described. Secondary symbionts are defined by being nonessential (they might be facultatively beneficial or parasitic). Like primary symbionts, they are also commonly transmitted vertically, but many also show horizontal transmission at varying rates (Tsuchida *et al*., 2002; Gottlieb *et al*., 2008; Moya *et al*., 2008) (Fig. 2). Some of the symbionts, such as *Wolbachia*, are able to manipulate host reproduction to spread through the population. Thus, *Wolbachia* infections in arthropods are generally considered to result in a parasitic association (Werren *et al*., 2008). Overall, microbe–host associations were established long ago (up to 200 million years in some cases) (Moran *et al*., 2008). This poses the question of how the animals can discriminate between harmful and beneficial microbes and whether the evolution of an adaptive immunity might have enabled vertebrates to evolve symbiotic interactions with resident microbes such as the gut microflora (McFall-Ngai, 2007).

The meeting 'Immunity and Symbiosis' of the European Union COST Action FA0701 'Arthropod Symbioses: from fundamental studies to pest and disease management' held in Aussois, France (27–29 May 2009), brought together scientists of different disciplines to inspire cooperative work to understand the molecular and immunological bases for such stable symbiotic interactions and to

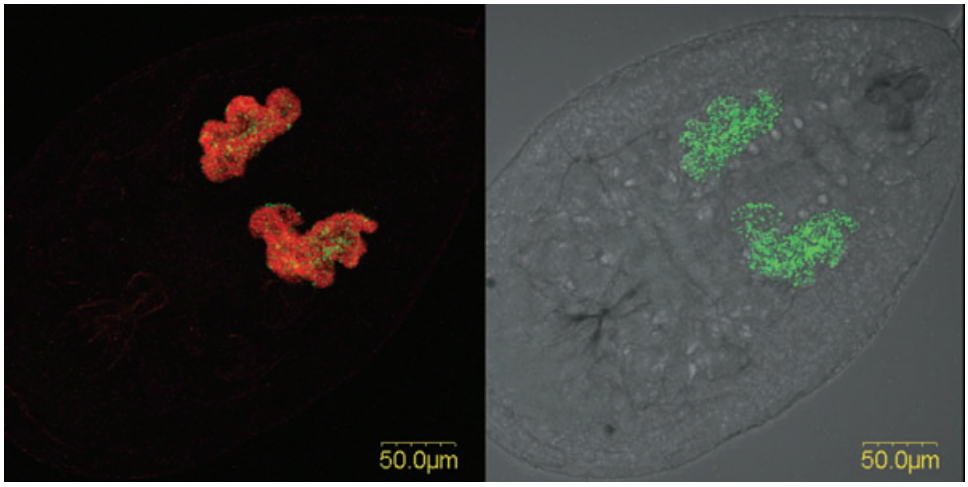

Fig. 2. Localization of primary and secondary endosymbionts in the nymph of a sweet potato whitefly *Bemisia tabaci*. The photograph shows a fluorescent *in situ* hybridization image of the bacteriomes containing both, the primary symbiont *Portiera* (red), a γ-Proteobacterium, and the secondary symbiont *Hamiltonella defensa* (green) (Gottlieb *et al*., 2008). Photograph kindly provided by Y. Gottlieb.

investigate possible contributions of symbiotic microorganisms to defence of their animal hosts against pathogens and parasites. It is hoped that this research field might lead to innovative strategies to defeat arthropod pests and to interfere with pathogen and parasite transmission by arthropod vectors. Here, we will briefly discuss a selection of the papers presented at the meeting.

MAMPs and PRRs involved in the establishment and maintenance of symbiosis

The symbiosis of the squid *Euprymna scolopes* and the bacterium *Vibrio fischeri* is a paradigmatic example of a highly evolved interaction, in which specific bacterial and host factors are important in the establishment of a symbiotic relationship (Ruby, 2008). This luminescent bacterium efficiently colonizes the light organ of young squids, and thereafter enters a circadian rhythm during which the vast majority of the bacteria is expelled from the organ every dawn, and bacterial titre then builds up again through the day. Thus, the light organ is re-populated from a small number of resident bacteria each day. Margaret McFall-Ngai (University of Wisconsin, Madison, USA) reported on the communication between the bacteria and the host that underlies these complex and highly specific interactions. The bacterial signals sensed by the host mainly consist of lipid A and a peptidogyclan-derived muramylpeptide, the tracheal cytotoxin (TCT). The latter is known as a potent virulence factor of the whooping cough agent *Bordetella pertussis* and of *Neisseria gonorrhoeae*, where it causes the loss of ciliated cells from either respiratory or fallopian tube epithelia respectively. *V. fischeri* releases significant amounts of this compound because it possesses altered lytic transglycosylases, and TCT, possibly in synergy with LPS, triggers light organ morphogenesis by regression of ciliated appendages in epithelium-lined crypts of the symbiotic organ, probably also involving host cell apoptosis (Koropatnick *et al*., 2004; Adin *et al*., 2009). This phenomenon is a nice example of the context-dependent action of bacterial signalling molecules, in which the same molecule favours or impedes the establishment of a mutualistic interaction. Interestingly, the MAMPs of *V. fischeri* might also be involved in the day–night control of the symbiosis. Just prior to dawn, the epithelial cells supporting the symbionts lose their polarity and become effaced, much like the cells of the mammalian trachea under the influence of the TCT produced by *B. pertussis*. This finding suggests a diurnal rhythm of host response to symbiont MAMPs. How such dynamics might be controlled will require further study. However, the diurnal cycle of the symbiosis might involve a switch between a kind of a pathogenic colonizing stage and a beneficial luminescent stage of the bacteria. Taken together, these data indicate that the host immune system is a key player in the establishment and maintenance of the symbiosis.

Chronic infections with symbiotic bacteria are very widespread in arthropods. The tsetse fly *Glossina morsitans* transmits the African trypanosomes. In addition, it harbours three vertically transmitted bacterial companions, the primary endosymbiont *Wigglesworthia glossinidia* present in bacteriocytes, the secondary facultative endosymbiont *Sodalis glossinidius* that is widely present in the gut, haemolymph and even in phagocytic haemocytes, and the reproductive parasite *Wolbachia* (Rio *et al*., 2006). Vertical transmission of *Sodalis* and *Wigglesworthia* occurs through milk glands, in which both symbionts live extracellularly and from which they can reach and invade 1st instar larvae of the viviparous animals (Fig. 3) (Attardo *et al*., 2008). Serap Aksoy (Yale School of Public Health, USA) reported about a new mechanism by which the secondary symbiont *Sodalis* can escape immune surveillance of the host. *S. glossinidius* is a close relative of *Escherichia coli* and has a quite large genome of about 4.1 Mb, probably reflecting recent entry into symbiosis. However, this genome has a coding capacity of only 49% and harbours 963 pseudogenes (Toh *et al*., 2006). In contrast to *E. coli*, *S. glossinidius* can multiply in the fly without causing harm to the animals, while *E. coli* injection in the hemocoel leads to sepsis and rapid death of the flies. A detailed analysis of the differences between *E. coli* and *Sodalis* identified the significance of variation of extracellular loops of the outer membrane protein A (OmpA) in determining the course of infection. A recombinant *E. coli* K12 strain with a deletion of its own *ompA* gene, but producing OmpA of *Sodalis*, was avirulent. Vice versa, *Sodalis* expressing the *E. coli ompA* gene was virulent. Subsequent analysis of the tsetse immune response to the different bacteria revealed that avirulent infections activated an immune response in several immune related genes, including genes for AMPs such as attacin and cecropin, while the immune response following exposure to the virulent bacteria carrying *E. coli* OmpA was relatively weak. In particular, cecropin levels were found to be much lower in flies infected with the virulent as compared with the avirulent bacteria. This suppression of cecropin expression might at least partially explain the pathogenic phenotype of *E. coli* K12 and *Sodalis* expressing the *E. coli ompA* gene, as cecropin is highly potent against Gram-negative bacteria, while *Sodalis* proved to be quite resistant to cecropin (Weiss *et al*., 2008). The tsetse homologue of the *D. melanogaster* PGRP-LB PRR GmPGRP-LB appears to play a key role in this immune response, as its expression after infection with virulent bacteria was much more pronounced than with avirulent bacteria. This PRR has direct amidase activity and negatively interferes with the Imd pathway, thus suppressing the immune response

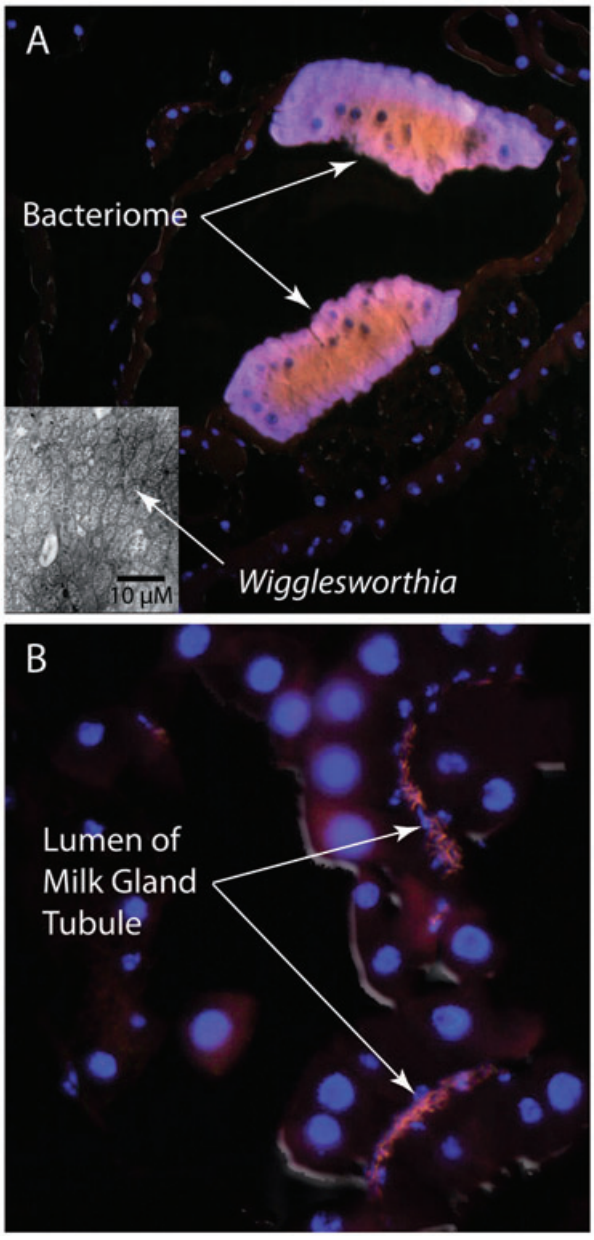

Fig. 3. Localization of *Wigglesworthia* in host cells and their transmission route to progeny in the Tsetse fly (*Glossina morsitans morsitans*).
A. Differentiated host epithelial cells (bacteriocytes) harbour the intracellular *Wigglesworthia*. Bacteriocytes form the bacteriome organ in the anterior midgut. Fluorescent *in situ* hybridization (FISH) was applied to tissue sections where DAPI staining shows cell nuclei (blue stain), and a 5′ Rhodamine-labelled bacterium-specific anti-16S probe shows *Wigglesworthia* localization (red/pink staining). The inset depicts an electron micrograph of *Wigglesworthia morsistans* in a bacteriocyte. The endosymbionts are surrounded by a two-layer membrane and lie free in the host cytoplasm. Bar = 10 µm.
B. FISH analysis of milk gland sections shows *Wigglesworthia* within the lumen. The bacteria are transmitted to intrauterine progeny via ingestion of maternal milk secretions. Photograph kindly provided by Serap Aksoy.

against the virulent bacteria. In fact, RNAi-induced knockdown of this gene led to a significantly reduced mortality of the host after infection with *E. coli*. Thus, variability in the OmpA protein might contribute to tolerance of the host versus its symbiotic bacteria (Weiss *et al*., 2008).

GmPGRP-LB also plays a role in the host interaction with the primary symbiont *Wigglesworthia*, as this PRR is expressed in tissues harbouring the endosymbiont and GmPGRP-LB levels increase during multiplication of the bacterium. Silencing of GmPGRP-LB by RNAi leads to a reduction in the number of *Wigglesworthia*, possibly by an increase in the expression of the antimicrobial effector attacin. The selective elimination of *Wigglesworthia* from animals by ampicillin treatment abolishes female fertility. Lack of the endosymbiont also reduced production of GmPGRP-LB and, unexpectedly, increases susceptibility to *Trypanosoma* infections, especially in older flies that are typically highly resistant to parasite infections (Pais *et al*., 2008). This indicates, on the one hand, that host immune responses are highly adapted to protect the essential symbiosis while, on the other hand, symbiosis-driven host responses might directly influence parasite resistance traits (Wang *et al*., 2009).

Protective symbiosis

The invasion of heritable endosymbionts into a host population can be driven by a contribution of endosymbionts to host fitness. In the case of primary endosymbionts of insects such as *Buchnera* in aphids or *Wigglesworthia* in tsetse flies, this increase in host fitness is mainly accomplished by nutritional upgrading of the specialized diet of the host animals, e.g. plant sap or vertebrate blood, by essential nutrients such as amino acids or vitamins (Zientz *et al*., 2004). However, an increasing number of observations indicate that such symbionts might also confer resistance to the host against natural enemies. A striking example was presented by Kerry Oliver (University of Georgia, Athens, USA). In addition to the primary endosymbiont *Buchnera*, aphids such as *Acyrthosiphon pisum* might carry one or more so-called secondary symbionts, most frequently *Hamiltonella defensa*, *Serratia symbiotica* and *Regiella insecticola*, members of the *Enterobacteriaceae*. These can be found both extracellularly, e.g. in the haemolymph, and intracellularly, both diffusely in the organism and in the bacteriocytes (Oliver *et al*., 2006). Aphids are frequent victims of parasitoid wasps, which lay their eggs in the body cavity of the animals. Natural populations of aphids show a strong variation in their resistance against parasite attack. Using experimental aphid lines that shared the same aphid genotype but harboured differed strains of *H. defensa*, it was shown that this symbiont confers protection against killing wasps as they develop within living aphids, and that

levels of protection varied depending upon bacterial strain. Further work using experimental populations showed that infection frequencies by this symbiont increase when parasites are present and decline again in the absence of the wasps (Oliver *et al*., 2008). After deposition of eggs by the wasp *Aphidius ervi* in the aphid *A. pisum*, the aphids are largely resistant to wasp-induced mortality and even reproduce after being parasitized. The presence of *S. symbiotica* also correlates with survival of the animals, but the parasitized animals do not reproduce. *H. defensa* is frequently infected with a bacteriophage called APSE (**A***cyrthosiphon* **p***isum* **s**econdary **e**ndosymbiont bacteriophage), the presence of which correlates with the survival of the animals after being parasitized (Moran *et al*., 2005). Most interestingly, this phage encodes several potential toxins known from vertebrate pathogens, including a cytolethal distending toxin, a Shiga-like toxin and a YD-repeat toxin (Degnan and Moran, 2008). These toxins might target eukaryotic tissue, in particular that of the developing wasp. The presence of potential antiparasitic factors within a phage genome of a secondary symbiont might indicate that ecologically important traits can be extensively transferred horizontally in aphid populations. The recent establishment of the genome sequence of *H. defensa* revealed the presence of additional factors possibly involved in the interaction with its host including a type III secretion system, the effectors of which might contribute either to establishment of the symbiosis or to beneficial effects of *H. defensa* (Degnan *et al*., 2009).

Another example of protective symbiosis involving bacteria was recently reported. *Wolbachia* is extremely widespread in arthropods, and up to 66% of all insects might be infected with this bacterium (Hilgenboecker *et al*., 2008). *Wolbachia* is able to manipulate host reproduction in different ways to increase its transmission to progeny, e.g. by cytoplasmic incompatibility or by feminization. Therefore, it is generally considered to have a parasitic interaction with its arthropod host (Werren *et al*., 2008). Karyn Johnson (University of Queensland, Brisbane, Australia) and Luis Teixeira (Instituto Gulbenkian de Ciencia, Oeiras, Portugal and University of Cambridge, UK) reported a new phenotype associated with *Wolbachia* presence in *D. melanogaster* and *Drosophila simulans*. It transpires that *Wolbachia* can confer resistance against *Drosophila* C virus, a natural RNA virus pathogenic to *Drosophila*, while animals cured of *Wolbachia* by tetracycline treatment are fully susceptible (Hedges *et al*., 2008; Teixeira *et al*., 2008). In *D. melanogaster*, *Wolbachia*-mediated viral resistance is associated with a 10 000-fold reduction in viral titre. Symbiont-mediated resistance in *D. melanogaster* extends to other RNA viruses, including Nora and Flock House virus, while protection is apparently not conferred against a DNA virus (Insect Iridescent virus 6). So far, it is not known how protection is achieved. The antiviral effect of different *Wolbachia* strains belonging to both supergroups A and B in *D. simulans* has been investigated. Interestingly, the level of antiviral protection and/or impairment of virus replication were quite variable, indicating that specific features associated with some *Wolbachia* strains are responsible for the antiviral phenotype.

Novel ways of pest management

Many arthropods are economically important pests themselves or are vectors of infectious agents causing important human diseases. Symbiont-based strategies are being evolved either to defeat the pest or to interfere with transmission of pathogenic microorganisms from the vector. Some of these strategies might become valuable tools in pest and disease management (Douglas, 2007). Several presenters discussed different approaches currently under investigation or development.

Already in widespread use is the 'Sterile Insect Technique', in which males of a pest species, e.g. the medfly, are sterilized by gamma-radiation and released into the environment, thereby leading to non-productive mating and reduction of the pest population (Dyck *et al*., 2005; Bourtzis, 2008). A problem with this approach, however, is the reduced fitness and mating efficiency of the treated males compared with their natural competitors. Boaz Yuval (Hebrew University of Jerusalem, Israel) reported on effects of irradiation of medflies on their gut microflora. In wild flies, the bacteria deposited during oviposition in the fruit, where they contribute to fruit decay, are identical to the gut microflora that is transmitted largely vertically. Among them are several potentially beneficial bacteria including diazotrophs (e.g. *Klebsiella oxytoca*) and pectin degraders (e.g. *Pectobacterium*) (Behar *et al*., 2008a). In the gut, a small but quite stable number of bacteria belong to a group of known entomopathogenic pseudomonads. Interestingly, irradiation in the industrial fly strain Vienna-8 leads to a strong shift in the microbial community in favour of the pseudomonads. This increase in potentially pathogenic bacteria might contribute to the decreased mating efficiency of the irradiated fly males. In fact, the addition of the potentially beneficial gut resident *K. oxytoca* to the post-irradiation diet improved the performance of the sterile males and might improve the quality of the anti-pest males in the future (Behar *et al*., 2008b; Ami *et al*., 2009).

The problem of reduced fitness of the sterile males has stimulated the development of an alternative, *Wolbachia*-based, population suppression approach called 'Incompatible Insect Technique' (Zabalou *et al*., 2004; 2009). Steven Sinkins (University of Oxford, UK) (Sinkins and Gould, 2006) discussed the potential use of *Wolbachia* for the control of vector-borne human diseases. The *w*MelPop strain from *D. melanogaster* has been success-

fully introduced into *Aedes aegypti* mosquitoes, which can transmit dengue virus. It reduced host lifespan by up to 50% (McMeniman *et al*., 2009). As many pathogens require quite a long period of development in the insect host, termed the 'extrinsic incubation period', before they can be efficiently transmitted to a new host, reduction of mean vector lifespan can significantly reduce pathogen transmission, without engendering very strong selection against the agent. As described above, the *w*MelPop strain has also recently been shown to provide protection against RNA viruses in *Drosophila* (Hedges *et al*., 2008; Teixeira *et al*., 2008). The combination of *Wolbachia*-induced lifespan shortening and reduced susceptibility to pathogens could provide a powerful tool to interfere with vector-borne diseases in the future.

Gut microbiota and gut homeostasis

Many arthropods live in a quite microbe-rich environment and ingest a great diversity of microorganisms, including potential pathogens. Moreover, the entry route of human pathogens or parasites into their animal vectors is oral, so these pathogens have to compete with the resident gut microbiota of the vector animal. Basic features of the innate immune system acting in particular in the gut of *D. melanogaster* were examined in several talks, and factors involved in the maintenance of a commensal gut microbiota were discussed. Apart from physicochemical and physiological properties, such as pH and gut peristalsis, additional important factors controlling the gut microbiota are lysozymes, reactive oxygen species (ROS) produced by the NADPH-dependent **du**al **ox**idase (DUOX), and local Imd-dependent production of AMPs, such as diptericin and attacin. Won-Jae Lee (Ewha Womans's University, Seoul, South Korea) described the constant activation of the Imd signalling pathway in the gut by the resident gut microflora. Interestingly, this activation does not lead to production of AMPs. The intestinal homeobox gene *Caudal* (*Cad*) is essential for this suppression of an excessive and permanent immune response against the commensal microbiota. *Cad* interferes with the activation of AMP genes mediated by an NF-κB-related transcription factor (Relish). Accordingly, RNAi-mediated inhibition of *Cad* expression caused overproduction of AMPs and a strongly altered commensal community structure in the gut. The changes in the gut microbiota lead to gut cell apoptosis and eventually to death of the flies. Pathological effects in the gut could not be observed after *Cad* knockdown in germ-free animals, suggesting that the altered composition of the gut microflora, and in particular the massive presence of a *Gluconobacter* strain, contributes to the pathological gut changes (Ryu *et al*., 2008). Besides the NF-κB-dependent immune reactions, an alternative antimicrobial pathway that involves the action of DUOX producing ROS seems to be important. DUOX is controlled by a phospholipase (PLCβ) (Ha *et al*., 2009). This shows that the gut epithelium of *D. melanogaster* has relevant antimicrobial defence strategies that are functionally autonomous from the previously described NF-κB-dependent mechanisms.

An important new issue regarding the maintenance of gut homeostasis during bacterial oral infection of *D. melanogaster* was reported by Bruno Lemaitre (Global Health Institute, Lausanne, Switzerland). The data reported suggest that, upon ingestion of bacteria, there is a delicate balance between cell damage and repair of the gut epithelium by rapid stem cell division. In particular, changes in the gut transcriptome after oral infection with a virulent *Erwinia carotovora* strain were investigated (Buchon *et al*., 2009). This strain was used because it is known to induce the Imd pathway in the gut strongly, but it does not kill adult flies. Among the induced genes was a large number encoding factors involved in antimicrobial defence and epithelial renewal, while factors involved in digestion were repressed. Most importantly, several components of the Imd, JAK/STAT and JNK and other signalling pathways involved in developmental processes were induced. This shows that the Imd pathway plays a major role in the regulation of immune genes in the gut. Interestingly, some drosomycin-like AMPs including dro3 were induced by the oral application of *E. carotovora*. The production of these factors appears to be specific for the gut, as they are not known to be induced in the fat body (the major immune responsive organ of insects). In the case of dro3, a direct connection to the JAK/STAT pathway could be revealed, thus demonstrating that this pathway, together with the Imd pathway, is directly involved in the control of AMPs in the gut. The JAK/STAT pathway is activated by Unpaired-3, a cytokine produced by enterocytes that are stressed or damaged during infection. A fascinating new finding is the observation that oral infection induced expression of a series of genes involved in cell repair and renewal in an Imd-independent manner. In fact, infection with *E. carotovora* leads to an increased cell death in the gut, mainly due to the production of ROS by DUOX (see above). To counteract this infection-related tissue destruction, multipotent intestinal stem cells present along the basal membrane in the gut are induced to proliferate, leading to an approximately 10-fold increase in dividing cells upon infection. Stem cell proliferation is also regulated by the JAK-STAT pathway, indicating a dual role of this pathway in both immunity and epithelial renewal. Similar conclusions were also reported by Dominique Ferrandon (Institut de Biologie Moléculaire et Cellulaire du CNRS, Strasbourg, France), who performed a genome-wide *in vivo Drosophila* RNAi screen with entomopathogenic *Serratia marcescens*, which causes disruption of the gut epithelium of the flies through

secretion of haemolysins (Cronin *et al*., 2009). Taken together, the data show that the gut homeostasis is important for the immune response, and that renewal of the gut epithelium is a direct consequence of bacterial infection and contributes to gut homeostasis. Thus, a direct connection between immunity and development is obvious.

Conclusions and future directions

A major challenge of symbiosis research in the future will be to bring together scientists of very different fields, as symbiosis research is multidisciplinary [involving microbiology, molecular biology, developmental biology, immunology and ecology (McFall-Ngai, 2008)]. Among the many questions that should be answered, just a few of particular importance with regard to the topic of the meeting are mentioned below.

We are far away from having a global overview of the microbial diversity associated with arthropods in symbiotic relationships. However, the current view indicates that some bacterial groups such as the γ-Proteobacteria and α-Proteobacteria, and including both pathogens and symbionts, are particularly prone to interact with animal tissues. The biological function of some primary symbionts in enriching the host diet is known but, in many cases, the biological basis of the symbiosis remains unclear and even in a 'simple' two-partner symbiosis symbionts might have more than one function. This lack of knowledge is especially dramatic for the role of the extremely widespread secondary (facultative) symbionts. In fact, multiple infections with vertically transmitted symbionts are frequent in arthropods, but little is known about the early events in the establishment of such multiple symbioses and their ecological and evolutionary importance (Vautrin and Vavre, 2009).

Moreover, we do not know much about the mechanistic basis required for the establishment of chronic mutualistic infections and how the host is able to discriminate between pathogens and beneficial symbionts, thereby 'defeating' pathogens but allowing beneficial symbionts to persist. One strategy would focus on the specific symbiont-bearing cells, the bacteriocytes. Analysis of immune responses and regulation in these cells would be of huge interest. In particular, it would be beneficial to identify particular intracellular receptors and pathways, and to understand evolutionary constraints that have shaped bacteriocyte tolerance to endosymbionts (Anselme *et al*., 2008). To accomplish this goal, basic investigations on the mode of action of innate immune systems of different non-model animals have to be performed and investigations into the possibly differential response of the innate immune system on symbionts and pathogens must be intensified. As invertebrates constitute the vast majority of animals on earth, it will be important to include as many organisms as possible in these studies to get insight into the vast potential of innate immune systems and into the evolution of immune-related processes in general (Jiravanichpaisal *et al*., 2006). Novel molecular techniques established only in the past few years, such as modern sequencing techniques and RNA interference, now allow us to extend research into symbiotic systems that were previously difficult to investigate, e.g. due to cultivation problems or the lack of genetic systems to manipulate the symbiotic partners. For instance, many invertebrate species are currently open to such a gene manipulation, including the weevil *Sitophilus*, in which RNAi was shown to knock down the bacteriocyte immune gene through a systemic response (Vallier *et al*., 2009).

There are now good opportunities for identifying novel factors relevant for the establishment and maintenance of symbiotic interactions through the characterization of the large number of hypothetical genes identified by searches for symbiosis-related differentially expressed genes in different organisms, e.g. by microarray analysis. To date, most hypothetical genes have been excluded from further analysis, and the major research focus is on those genes with known or deduced function. The comparison of the data sets obtained in screens with different organisms under similar conditions might lead to the identification of hypothetical genes that are conserved between the organisms that, as indicated by their expression patterns, are evidently related to symbiosis. As recently suggested, the evolutionary conservation of such hypothetical genes might indicate their basic function in symbiosis and might thus encourage the in-depth investigation of such genes in the future (Chun *et al*., 2008).

A newly developing field is based on the discovery that microbial symbionts might contribute to defence against pathogens and natural host enemies, as exemplified by *H. defensa* and some *Wolbachia* strains. So far, we do not know how widespread symbiosis-based pathogen and enemy resistance are or how underlying molecular mechanisms of protection operate, but these discoveries urge us to consider symbionts as an additional component of the host immune system. Novel symbiont-based strategies aiming in the control of agricultural pests or disease vectors will strongly profit from basic research in symbiont–host interactions and in innate immune systems of invertebrates; however, the proper regulatory framework should first be established before any symbiont-based pest and/or disease control strategy is applied.

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