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Infection in a dish: high-throughput analyses of bacterial pathogenesis

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Running head: Infection in a dish

SUMMARY

Diverse aspects of host-pathogen interactions have been studied using non-mammalian hosts such as *Dictyostelium discoideum*, *Caenorhabditis elegans*, *Drosophila melanogaster* and *Danio rerio* for more than 20 years. Over the last two years, the use of these model hosts to dissect bacterial virulence mechanisms has been expanded to include the important human pathogens *Vibrio cholerae* and *Yersinia pestis*. Innovative approaches using these alternative hosts have also been developed, allowing new antimicrobials to be isolated through screening large libraries of compounds in a *C. elegans-Staphylococcus aureus* infection model. Host proteins needed by *Mycobacterium* and *Listeria* during their invasion and intracellular growth have been uncovered by high-throughput dsRNA screens in a *Drosophila* cell culture system, while immune evasion mechanisms deployed by *Pseudomonas aeruginosa* during its infection of flies have been identified. Together, these reports further illustrate the potential and relevance of these non-mammalian hosts for modelling diverse aspects of bacterial infection in mammals.

INTRODUCTION

The use of mammalian models to identify and understand virulence factors of human pathogens is indispensable. Alternative models, such as the amoeba *Dictyostelium discoideum*, the nematode *Caenorhabditis elegans*, the insect *Drosophila melanogaster* and the fish *Danio rerio* are, however, complementary systems for such studies [1-5]. This is possible because many human pathogens are of low species specificity and can infect hosts ranging from insects and nematodes to fish, as well as other mammals. They rely on universal virulence factors that are involved in the infection process regardless of the host. These can be identified and characterised using genetically tractable and inexpensive non-mammalian models. In addition, the molecular and genetic tools developed with these simple organisms combined with their well-studied cellular biology and/or immunology allow one to decipher the complex interactions between host and pathogen.

The four organisms listed above have in common many characteristics, such as ease of culture and full sequenced genomes, that make them very useful as model hosts [1]. These alternative hosts are being used for approaches as diverse as testing the virulence of chosen pathogen mutants [6,*7], screening large banks of pathogen mutants for those with attenuated virulence [8, *9, *10] or dissecting the host mechanisms involved in pathogen invasion and intracellular replication [**11, *12, *13].

In addition, they have unique features relevant to the study of specific aspects of host-pathogen interactions. The amoeba *D. discoideum* is a professional phagocyte that can be used to decipher the molecular basis of phagocytosis and phagosome maturation [4]. Additionally, it can give insights into how certain intracellular bacterial pathogens survive in the phagolysosome [14]. The fly *D. melanogaster* possesses a very well-studied innate immunity [15] that has contributed to the understanding of immune mechanisms in mammals. More

recently, it has been used to analyze the mechanisms used by pathogens to evade the host immune system [*16, 17, **18]. Finally, genetic screens for bacterial virulence genes in a vertebrate with a fully developed immune system [19] are possible with the fish *D. rerio*. This review focuses on recent work with the alternative model hosts *D. discoideum*, *C. elegans*, *D. melanogaster* and *D. rerio* in these new investigative paradigms.

New infections modelled with alternative hosts.

An increasing number of human bacterial pathogens are being tested in non-mammalian hosts in order to study conveniently their virulence. In addition to established models such as *Pseudomonas aeruginosa* [20,21], *Salmonella typhimurium* [22-24] or *Serratia marcescens* [25,26], several pathogens including *Listeria monocytogenes* [27,28], *Yersinia pestis* (see below) and *Vibrio cholerae*, the causal agent of cholera, have recently been added to the list of micro-organisms able to cause lethal infection of the nematode and the fly.

In humans, expression of Cholera toxin (CT) by *V. cholerae* provokes a rise in cAMP in the intestinal epithelium, the opening of ion channels and consequently loss of water into the intestinal lumen. In mice, this secretory diarrhea can be successfully treated with the channel blocker clotrimazole. It has now been reported that oral *V. cholerae* infection of the fruit fly leads to the death of the animals in a manner somewhat similar to that observed in humans, including rapid weight loss [*7]. CT is required for full virulence in the fly model and, remarkably, flies with loss-of-function mutations in genes encoding homologues of the known targets of CT resist infection. Furthermore, clotrimazole can help cure flies infected with *V. cholerae* [*7].

During the lethal colonization of the *C. elegans* intestine by *V. cholerae*, on the other hand, CT does not appear to play an important role [6]. But, using a reverse genetic approach, Vaitkevicius and colleagues demonstrated that the quorum sensing regulated protease PrtV is

essential for this killing. Moreover, they obtained data [6] suggesting that this protease is important for *V. cholerae* in its natural niche [29] for its resistance to the marine plankton that graze on it. Finally, they measured an increased IL-8 secretion in human epithelial intestinal cells exposed to a *V. cholerae prtV* deletion mutant, compared to the parental strain, suggesting a role for this protease in modulating (directly or indirectly) the host response in vertebrates [6].

Together, these reports illustrate to what extent nematode and fly can be relevant for the study of the causative agent of cholera. Importantly, the work by Blow and colleagues offers the perspective of using *Drosophila* to screen for chemicals that inhibit CT *in vivo*, following a precedent set by the Ausubel lab using *C. elegans* [**30].

In vivo screens for new antimicrobials

The massive use of antibiotics combined with the high adaptation capacity of bacteria has created the public-health problem of human pathogens resistant to multiple antibiotics. Therefore, there is a real need for new antibiotic molecules. Moy and colleagues cleverly used an infection system involving a *C. elegans* immunocompromised mutant and *Enterococcus faecalis* [**30] to screen for synthetic (6,000) and natural (>1,000) molecules that promoted host survival (FIGURE 1). This *in vivo* screen not only permitted the identification of 8 molecules affecting bacterial growth *in vitro* (MIC < 35 µg/ml), but also of 8 other products either impairing pathogen virulence or boosting host innate immunity in the absence of significant *in vitro* activity (MIC > 125 µg/ml) [**30]. Even though the efficiency and toxicity of the identified molecules does need to be tested in mammals, this system represents a very promising screening platform to identify *in vivo* new antibacterial molecules. A similar system involving flies to identify antifungal drugs is also being developed [31,32].

Random screens for the identification of bacterial virulence genes

Three recent reports using *D. discoideum*, *C. elegans*, and *D. rerio* as hosts to screen bacterial mutant libraries of *Klebsiella pneumoniae*, *Y. pestis*, and *Streptococcus iniae*, respectively, have further strengthened the relevance of these simple hosts.

K. pneumoniae is an important human pathogen that, as its name suggests, causes pneumonia. Its interaction with alveolar macrophages can be modelled using *D. discoideum* as a surrogate phagocyte. *D. discoideum* is normally able to feed on wild-type *Klebsiella*. Benghezal and colleagues elegantly combined the genetics of *D. discoideum* and the genetics of *K. pneumoniae*. They first identified a new gene (*phg1*) [33] rendering the amoeba especially susceptible to infection and unable to grow on *Klebsiella*. Then, they isolated *Klebsiella* mutants that supported the growth of the *phg1* mutant amoeba [*10]. Among the mutated bacterial genes were several required for LPS and amino-acid biosynthesis. They tested several of the isolated bacterial mutants in a mouse pneumonia model and found an attenuation of virulence [*10].

The genetic manipulation of both host and pathogen allowed them to create a 2D virulence array showing that distinct groups of host genes are necessary to resist infection by various bacterial pathogens and mutants (FIGURE 2). They were also able to demonstrate conservation of both virulence factors and defence genes since *Drosophila phg1* mutants are more susceptible to *K. pneumoniae* infection [*10].

Y. pestis, the causative agent of plague, can form a biofilm that is important for its dissemination via its vector, the flea. A *Y. pestis* biofilm can also accumulate on the head of *C. elegans*, and this is clearly a more accessible model to study biofilm function than looking in the gut of the flea [34]. But as biofilm formation is only one aspect of *Y. pestis* pathogenicity, Styer and colleagues developed a nematode-based infection system to identify *Y. pestis* virulence genes not related to biofilm formation [*9]. They showed that a biofilm-deficient mutant of *Y. pestis* colonises the intestine of *C. elegans* and provokes an early death.

They used this infection model to screen a bank of *Y. pestis* mutants for those with attenuated virulence in the worm. Remarkably, despite the differences between nematodes and mammals, they identified two genes whose products are necessary for induction of mouse death in an intranasal mouse model of *Y. pestis* pathogenesis, genes that had previously not been implicated in *Y. pestis* virulence [*9].

S. iniae is a bacterial pathogen able to infect fish and humans. In order to analyze the interaction between streptococcal pathogens and their natural hosts, Miller and colleagues created a bank of bacterial mutants and screened it using zebrafish [8]. They wished to identify bacterial mutants specifically deficient in their capacity to disseminate in the brain. To facilitate the screening process, they used a Signature-Tagged Mutagenesis strategy [35] that permitted the analysis of fish co-infected by a pool of 12 distinct mutant strains. Doing so, they screened 1,128 signature-tagged transposon bacterial mutants and determined which bacterial mutants were not present in brain extracts from infected fish. Interestingly, 7 out of the 41 bacterial mutants isolated had transposon insertions in genes potentially coding for surface polysaccharides, major components of the capsule. Finally, using the bacterial mutants they isolated, they showed in a human whole blood assay for phagocytosis that *S. iniae*'s capsule is involved in invasion and survival in human macrophages [8].

These three studies further validate the use of non-mammalian hosts for large-scale screens to identify bacterial virulence genes relevant to infection in mammals. Moreover, the genetic manipulation of the host as exemplified by the work of Benghezal *et al.*, expands the range of models available for this kind of screening approach, in a manner reminiscent of the directed modification of mice, via transgenesis [36] or the creation of human-mouse chimeras [37], but without any of the ethical concerns.

Identification of host molecules required for pathogenesis and how the pathogen evades the immune system.

The host factors involved in the infection processes are not restricted to “immunity” genes such as those coding for interleukins or Toll-like receptors (TLRs). This is especially the case for intracellular bacterial pathogens that have to enter the cell and avoid being degraded in phagolysosomes. Therefore, intracellular bacteria have developed many ways to hijack the endocytic or phagocytic routes [38,39]. Macrophages are often confronted by intracellular pathogens as they are professional phagocytes. Three recent studies used the *Drosophila* S2 macrophage-like cell line to perform large scale RNAi screens to identify host factors required for entry and survival of intracellular bacterial pathogens.

The first two analyses combined automated microscopy with the use of GFP-tagged *Mycobacterium fortuitum* [*13] or *Listeria monocytogenes* [*12] to screen a bank containing 21,300 dsRNAs (targeting >95% of annotated *Drosophila* genes in a redundant fashion). They showed that factors involved in vesicle trafficking and actin cytoskeleton organization are necessary for internalization and intracellular survival of these two pathogens. Moreover, they identified a *Drosophila* homologue of the scavenger receptor CD36 as being crucial for *L. monocytogenes* and *M. fortuitum* entry into the S2 cells, while being dispensable for phagocytosis in general [*12,*13]. Based on these observations, the study was extended to mammalian cells and new roles in bacteria uptake described for members of the CD36 family. This work also highlighted a role for autophagy in the control of *L. monocytogenes* infection [*12].

In contrast to these two studies that used automated microscopy, a third study was performed manually [**11]. In this painstaking project, interest was focused especially on the interaction between the *L. monocytogenes* toxin listeriolysin O (LLO) and host factors that allow the bacteria to escape from the phagosome. The authors combined RNAi against the host with bacterial mutants for LLO. In a first set of experiments, they used an LLO-deficient bacterial strain and screened for dsRNAs that restored the capacity of these mutants to escape

into the cytoplasm. The corresponding genes would be expected to be elements of the host pathways targeted by LLO. In a second set of experiments, they used a bacterial mutant producing a LLO toxin lacking a PEST sequence (and thus with a longer half-life). They screened for dsRNAs that rendered S2 cells more susceptible to this stable toxin to determine which host enzymes control LLO toxicity. Based on their results, they propose a model in which the pore-forming LLO inserts into the membrane of the *L. monocytogenes*-containing phagosome, thus impairing its acidification and maturation. Concerning the host's control of LLO toxicity, their screen identified SPT, an enzyme necessary for sphingolipid metabolism, as a key factor for host resistance [**11].

The experimental systems described in these three reports can thus be used to shed light on the complex interactions between the host and an intracellular pathogen that are both fighting for their survival. But just as is the case for any model system, the results come with a number of caveats. It is well known that a dsRNA can interfere with off-target genes and so generate false positive results [40]. And conversely, important genes can be missed if they are not expressed in or on S2 cells, as is indeed the case for some receptors involved in phagocytosis (Istvan Ando and Dan Hultmark, personal communication). Nevertheless, in the long term, by combining large-scale screens in the host and the pathogen, it will be possible to define a host-pathogen interactome (Figure 2) [41].

Extracellular bacterial pathogens are usually not able to survive phagocytosis. Many, however, have developed strategies to counteract the humoral arm of the host immune system. A handful of recent articles have demonstrated that *Pseudomonas* infection of *D. melanogaster* is a most suitable system to study the host immune response and to uncover the strategies used by the pathogen to elude defence mechanisms. In one article, the role of the *Pseudomonas* exotoxin ExoS was directly addressed by ectopic expression in the eye or by ubiquitous expression of this bacterial protein in the fly [17]. The authors showed with

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these transgenic systems that ExoS inhibits the activity of a host RhoGTPase *in vivo* and that ubiquitous ExoS expression impairs the phagocytic capacity of fly macrophages without affecting anti-microbial peptide gene induction [17]. In a complementary study, Liehl and colleagues used host and pathogen mutants to demonstrate that the *Pseudomonas* AprA metalloprotease directly degrades fly antimicrobial peptides [18]. This protease thereby acts as a virulence factor by enhancing bacterial survival within the host body fluid. In addition to these reports, Apidianakis and colleagues compared microarray results from flies infected by virulent or avirulent *P. aeruginosa* strains [16]. Strikingly, this analysis revealed an as yet uncharacterised mechanism used by *P. aeruginosa* in the early phases of the infection to limit *Drosophila* antimicrobial genes expression at the transcriptional level.

Together, these studies illustrate the potential use of genetically tractable non-mammalian hosts, with characterized immune systems, to decipher the mechanisms pathogens employ to evade the host immune system. As exemplified above, it is possible to have a global approach and/or to address precisely the role of a specific bacterial protein.

CONCLUSIONS

Molecular and physiological conservation within different bacteria and across eukaryotic species renders the study of host-pathogen interaction using non-mammalian models especially attractive. Moreover, it is likely that many of the virulence mechanisms that pathogens use during their infection of humans in fact evolved since they favour survival in the natural ecological niche and so are best studied using their natural predators, such as *D. discoideum* and *C. elegans*. After a period when these model systems were used in essentially one-sided approaches (e.g. screening banks of bacterial mutants for virulence genes or identifying the host targets of bacterial virulence factors), more and more studies are now exploiting a combination of bacterial and host genetics to address the molecular basis of

pathogenicity and defence. The future promises to reveal details of the intimate but deadly dance between pathogen and host that has been going on since the birth of eukaryotes.

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The authors used a bank of 7,216 dsRNA to identify host factors involved in pathogenesis of *Drosophila* S2 cells infected by *L. monocytogenes*. In order to uncover the host pathways targeted by the bacterial LLO toxin and host mechanisms protecting the cell from this toxin, they combined this large-scale approach with bacterial mutants. They identified host proteins not previously involved in *Listeria* pathogenesis and enzymes involved in LLO-detoxification.

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In order to uncover host factors necessary for intracellular bacterial infection, these two papers used genome-wide RNAi bank, a *Drosophila* macrophage-like cell line, automated microscopy and GFP-tagged *L. monocytogenes* [*12] or *M. fortuitum*. [*13] Several hundred dsRNA altering the progression of infection were identified. The results obtained in the two screens were directly compared [*12]. Peste, a member of the CD36 family was found to be crucial for the entry of both bacteria into cells.

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FIGURES

Figure 1

Protocol used by Moy and colleagues [**30] to screen *in vivo* for new antimicrobial compounds using an established *C. elegans-E. faecalis* infection system. After culture and amplification of nematode numbers on growth plates (seeded with the *Escherichia coli* strain OP50) synchronised populations of worms are transferred to infection plates, seeded with *E. faecalis* strain MMH594. After 8 hours, worms are washed off the plates and approximately 25 individuals added to each well of a 96-well microtitre plate and then assayed for their survival. Compounds or extracts that extended worm survival by 2- to 3-fold were selected for further analyses. While this screen was carried out manually, the availability of the Union Biometrica Biosort (<http://www.unionbio.com/products/copas2.html>) allows automation of the different steps.

Figure 2

Hypothetical host-pathogen two dimensional array inspired by data from Benghezal *et al.* [*10]. The ability of host mutants to resist (green) or to be killed (red) by different bacterial strains and bacterial mutants is indicated. Gene names are arbitrary with *hrg* and *pvf* for Host Resistance Gene and Pathogen Virulence Factor, respectively. Based on this matrix, it can be speculated that *hrg-1* encoded protein is specifically involved in a mechanism necessary for host resistance to bacterial virulence factors encoded by *pvfB* and *pvfC*. The *hrg-3-pvfD* interaction would correspond to the case described by Liehl and colleagues [**18] with *hrg-3* and *pvfD* being the *Drosophila Imd* and *Pseudomonas aprA* genes, respectively. Finally, HRG-2 and HRG-4 can be host proteins necessary for bacterial invasion by pathogen C and pathogen B and C, respectively, corresponding to the observations described in the reports by Philips *et al.* and Agaisse *et al.* [*12, *13].

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Figure 1



