ELSEVIER

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

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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 past 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, enabling the isolation of new antimicrobials through screening large libraries of compounds in a *C. elegans Enterococcus faecalis* infection model. Host proteins required by *Mycobacterium* and *Listeria* during their invasion and intracellular growth have been uncovered using high-throughput dsRNA screens in a *Drosophila* cell culture system, and 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 many facets of bacterial infection in mammals.

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Current Opinion in Microbiology 2007, **10**:10–16

This review comes from a themed issue on
Host–microbe interactions: bacteria
Edited by Pamela Small and Gisou van der Goot

Available online 18th December 2006

1369-5274/$ – see front matter

DOI 10.1016/j.mib.2006.12.001

Introduction

The use of mammalian models to identify and understand the 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* can be complementary systems for such studies [1–5]. This is possible because many human pathogens have a 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 that have been developed for use with these simple organisms, combined with their well-studied cellular biology and/or immunology, enable one to decipher the complex interactions between host and pathogen.

The four organisms listed above have many factors in common that make them very useful as model hosts, such as the availability of their fully sequenced genomes and their ease of culture [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 that are 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 conveniently study 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 microorganisms that are capable of causing 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 diarrhoea can be successfully treated with the channel-blocker clotrimazole. It has now been reported that oral infection of the fruit fly by *V. cholerae* 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*, however, CT does not appear to play an important role [6]. But, using a reverse genetic approach, Vaitkevicius *et al.* [6] demonstrated that the quorum sensing regulated protease PrtV is essential for this killing. Moreover, they obtained data suggesting that this protease is important to *V. cholerae* in its natural niche [29] for its resistance to the marine plankton that graze on the bacterium. Finally, they measured an increased interleukin-8 (IL–8) secretion in human epithelial intestinal cells exposed to a *V. cholerae prtV* deletion mutant, compared to that of 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 *et al.* [7•] are compatible with the idea of using *Drosophila* to screen for chemicals that inhibit CT *in vivo*, following a precedent set by the Ausubel laboratory [30••], using *C. elegans*.

In vivo screens for new antimicrobials

The massive use of antibiotics, combined with the high adaptation capacity of bacteria has created a huge public health problem with many human pathogens becoming resistant to multiple antibiotics. Therefore, there is a real need for new antibiotic molecules. Moy *et al.* [30••] cleverly used an infection system involving a *C. elegans* immunocompromised mutant and *Enterococcus faecalis* to screen thousands of synthetic and natural molecules to find those that promoted host survival (Figure 1). This *in vivo* screen not only permitted the identification of eight molecules that affect bacterial growth *in vitro* (minimum inhibitory concentration [MIC] $<35\ \mu g\ ml^{-1}$) but also of eight other products that either impair pathogen virulence or boost host innate immunity in the absence of significant *in vitro* activity (MIC $> 125\ \mu g\ ml^{-1}$) [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 be used to identify antifungal drugs is also being developed [31,32].

Random screens for the identification of bacterial virulence genes

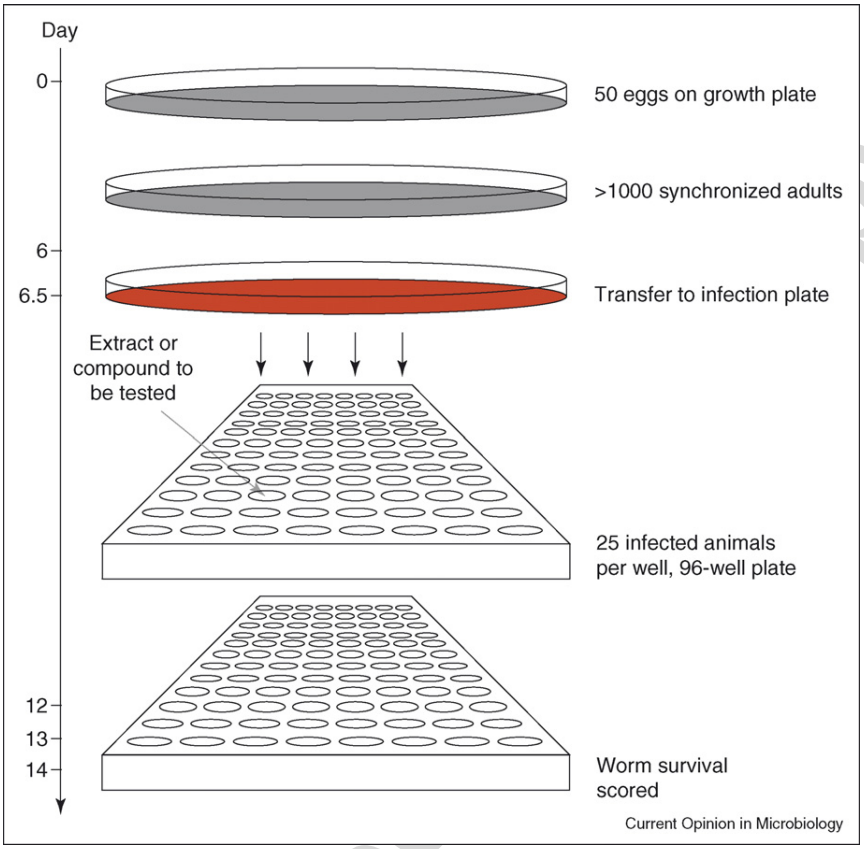
Three recent reports [8,9•,10•] using *D. discoideum*, *C. elegans* and *D. rerio* as hosts to screen bacterial mutant libraries of *Klebsiella pneumoniae*, *Y. pestis* and *Streptococcus iniae*, respectively, have further strengthened the relevance of these simple hosts.

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*. Cosson and colleagues [10•,33] elegantly combined the genetics of *D. discoideum* and the genetics of *K. pneumoniae*. They first identified a new gene (*phg1*) that, when mutated, rendered the amoeba especially susceptible to infection and unable to grow on *Klebsiella*. They then isolated *Klebsiella* mutants that supported the growth of the *phg1* mutant amoeba: among the mutated bacterial genes were several that were required for biosynthesis of lipopolysaccharides and amino acids. 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 enabled the authors 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 because *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 dissemination by 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 for studying biofilm function than is looking in the gut of the flea [34]. As biofilm formation is only one aspect of *Y. pestis* pathogenicity, Styer *et al.* [9•] developed a nematode-based infection system to identify *Y. pestis* virulence genes not related to biofilm formation. They showed that a biofilm-deficient mutant of *Y. pestis* colonises the intestine of *C. elegans* and provokes an early death of the host. They used this infection model to screen a bank of *Y. pestis* mutants for those with attenuated virulence in the nematode. Remarkably, despite the differences between nematodes and mammals, they identified two genes necessary for full virulence in an intranasal mouse model of *Y. pestis* pathogenesis, genes that had previously not been implicated in *Y. pestis* pathogenicity [9•].

Figure 1



Protocol used by Moy *et al*. [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 h, 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 twofold to threefold after 6–8 day's culture were selected for further analyses. Whereas this screen was carried out manually, automation of different steps is possible with tools such as Union Biometrica Biosort (http://www.unionbio.com/products/copas2.html). It is important to note that similar screens for antimicrobial compounds can be designed using *C. elegans* and other pathogens. The time when worm survival is scored will vary depending on the pathogen used.

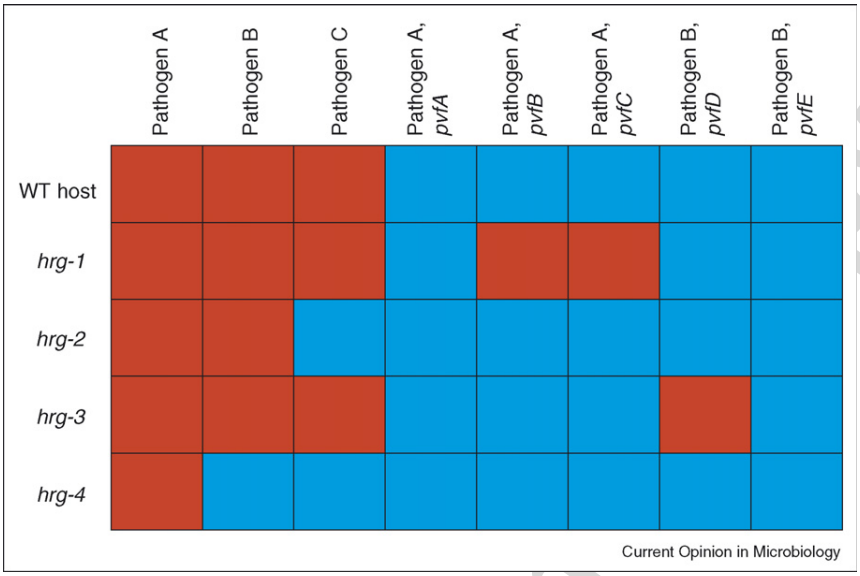
S. iniae is a bacterial pathogen that is able to infect fish and humans. To analyze the interaction between streptococcal pathogens and their natural hosts, Miller *et al*. [8] created a bank of bacterial mutants and screened it using zebrafish. They wished to identify bacterial mutants specifically 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 1128 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 required for the production of capsular polysaccharides. Finally, using the bacterial mutants they isolated, they showed in a human whole blood assay for phagocytosis that the capsule of *S. iniae* 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*. [10•], expands the range of models available for this kind of screening approach, in a manner reminiscent of the directed modification of mice, through trangenesis [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

Figure 2



Hypothetical host–pathogen 2D array inspired by data from Benghezal *et al*. [10•]. The ability of host mutants to resist (blue) or their susceptibility to (red) 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* 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 *et al*. [18••] with *hrg-3* and *pvfD* being the *Drosophila Imd* and *Pseudomonas aprA* genes, respectively. Finally, *hrg-2* and *hrg-4* could encode host proteins necessary for bacterial invasion by pathogen C and pathogens B and C, respectively, corresponding to the observations described in the reports by Philips *et al*. [12•] and Agaisse *et al*. [13•].

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 because they are professional phagocytes. The *Drosophila* S2 macrophage-like cell line has now been used in three large-scale RNA interference (RNAi) screens in order to identify host factors required for entry and survival of intracellular bacterial pathogens [11••,12•,13•]. The first two analyses combined automated microscopy with the use of green fluorescent protein (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 Peste (French for 'plague'), a *Drosophila* homologue of the scavenger receptor CD36, as being crucial for entry of *L. monocytogenes* and *M. fortuitum* into the S2 cells, whilst being dispensable for phagocytosis in general [12•,13•]. On the basis of these observations, the study was extended to mammalian cells and new roles in uptake of bacteria were 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, which 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 enable the bacteria to escape from the phagosome. The authors used RNAi to inactivate host genes and combined this with bacteria mutated in LLO. In a first set of experiments, they used an LLO-deficient bacterial strain unable to leave the phagosomes of normal cells 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 that lacks the PEST sequence which normally makes the protein relatively unstable. They screened for dsRNAs that rendered S2 cells more susceptible to this stable toxin in order to determine which host enzymes control LLO toxicity. On the basis of their results, they proposed 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 serine palmitoyl-CoA transferase (SPT), which is 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 several caveats. It is well known that a dsRNA can interfere with off-target genes and so generate false positive results [40]. 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 infection of *D. melanogaster* with *Pseudomonas* 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 [17], the role of the *Pseudomonas* exotoxin ExoS was directly addressed by expressing this toxin either ectopically in the eye or ubiquitously throughout the fly. The authors showed with these transgenic systems that ExoS inhibits the activity of a host Rho GTPase *in vivo* and that ubiquitous ExoS expression impairs the phagocytic capacity of fly macrophages without affecting induction of antimicrobial peptide genes [17]. In a complementary study, Liehl *et al.* [18••] used host and pathogen mutants to demonstrate that the *Pseudomonas* AprA metalloprotease directly degrades fly antimicrobial peptides. This protease thereby acts as a virulence factor by enhancing bacterial survival within the host body fluid. In addition to these reports, Apidianakis *et al.* [16•] compared microarray results from flies infected by virulent or avirulent *P. aeruginosa* strains. Strikingly, this analysis revealed an as yet uncharacterised mechanism used by *P. aeruginosa* in the early phases of the infection to limit expression of *Drosophila* antimicrobial genes 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 host defenses. As exemplified above, it is possible to have a global approach and/or to precisely address the role of a specific bacterial protein.

The principal drawbacks with these models are associated with bacterial physiology and the specificity of certain bacterial pathogens for their mammalian host. For instance, some virulence genes involved in mammalian pathogenesis are only expressed at 37 °C, whereas not all the model animals described in this review can be grown at this temperature [5]. Moreover, *C. elegans* does not possess macrophage-like cells [42] and some receptors necessary for the engulfment of intracellular bacterial pathogens in mammalian cells are absent from the surface of non-mammalian cells, thus limiting the utility of simple organisms for the study of intracellular pathogenesis. The same is true concerning mammalian signalling pathways specifically targeted and hijacked by some pathogens (e.g. although it possesses one TLR, NF-κB transcription factors, crucial for mammalian immunity, are not present in *C. elegans* [43]).

Conclusions

Although evolutionary divergence from mammals can limit the pertinence of simple model animals, the papers described in this review demonstrate that there is a wide-spread conservation of host–pathogen interactions at the molecular and physiological levels. In the light of this, the phylogenetic distance between a model system and mammals can even be considered a boon because conserved interactions are frequently the most important. Therefore, given the practical advantages associated with their use, non-mammalian models are increasingly being recognized as attractive alternatives to more traditional models [5]. Moreover, it is probable that many of the virulence mechanisms that pathogens use during their infection of humans in fact evolved because they confer a survival advantage 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.

Update

It has recently been shown using *C. elegans*, the *P. aeruginosa* strain PA14 and a PA14 *gacA* mutant (that is highly attenuated) that the intrinsic virulence of PA14 is a major elicitor of the host's innate immune response [44•]. In addition, by using the same animal model and by comparing the genome of the *P. aeruginosa* strain PA14 with that of PA01, it has been demonstrated that *Pseudomonas* virulence is multifactorial and necessitates the combinatorial action of multiple virulence factors that interact in a distinct manner, depending on the bacterial genetic background [45•].

Acknowledgements

We thank Pierre Golstein for helpful criticism. Work in the authors' laboratory is supported by the Fondation Recherche Médicale, INSERM, the CNRS, the French Ministry of Research, Marseille-Nice Génopole, the Réseau Nationale des Génopoles, the European Union and the French National Research Agency (ANR).

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- •• of outstanding interest

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See annotation for [13•].

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To uncover host factors necessary for intracellular bacterial infection, these two papers used a 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 that altered 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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