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Christon J. Hurst *Editor*

The Rasputin Effect: When Commensals and Symbionts Become Parasitic

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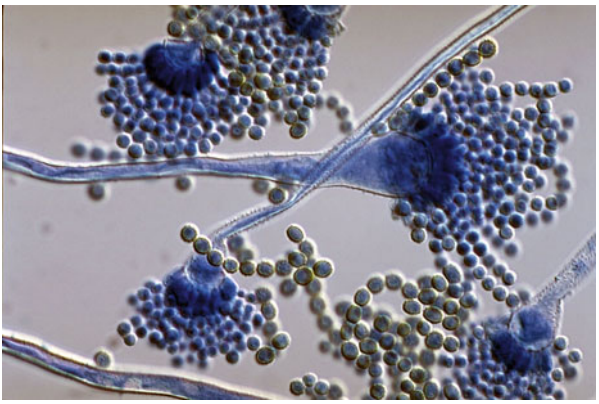

Aspergillus flavus. Courtesy of Hossein Mirhendi

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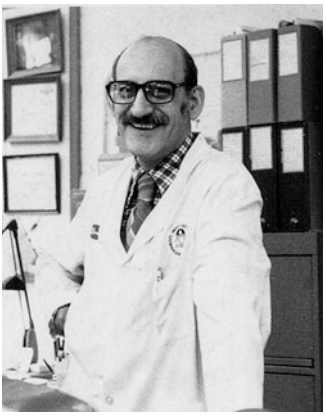
Dedication

I met Donald Berman through one of his daughters when both she and I were undergraduate students at the University of Cincinnati. She knew that I was interested in studying viruses and told me her father did that kind of work. I met her father soon afterwards and he undertook the task of encouraging my pursuit of virology. One summer, I took some time away from my undergraduate job of making flavorings and fragrances to instead learn from Don about propagating polioviruses and performing viral plaque assays. My first day spent working with him was interesting in two ways, one of which held true for the rest of my career in science. That afternoon we had birthday cake because it happened to be the birthday of someone in the virology group. Don and I stayed and worked far beyond normal quitting time on that day, not because we had been eating cake but simply because laboratory research always seems to take longer than you optimistically anticipate. The concept of having cake in the afternoon turned out not to be a normal part of the work days in science. My understanding that laboratory research always took more time than anticipated did continue to hold true for all of the years that were to follow.

I spent many happy hours of my undergraduate years working with Don, and I appreciate that his family always welcomed me very kindly into their home as if I naturally belonged there. Don also helped by guiding me to graduate school for studying virology. After I finished my formal education, Don helped me to find a job where he was employed, and I then happily anticipated seeing him each workday for perhaps an additional 15 years until he retired. I wish that I could relive the summer when I learned to do plaque assays by working alongside Don. Instead, since reliving that summer is not possible, I will derive pleasure from my remembrances and in gratitude I dedicate this book to him.



Donald Berman (1925–2011)

Series Preface

The light of natural philosophy illuminates many subject areas including an understanding that microorganisms represent the foundation stone of our biosphere by having been the origin of life on Earth. Microbes therefore comprise the basis of our biological legacy. Comprehending the role of microbes in this world which together all species must share, studying not only the survival of microorganisms but as well their involvement in environmental processes, and defining their role in the ecology of other species, does represent for many of us the Mount Everest of science. Research in this area of biology dates to the original discovery of microorganisms by Antonie van Leeuwenhoek, when in 1675 and 1676 he used a microscope of his own creation to view what he termed "animalcula," or the "little animals" which lived and replicated in environmental samples of rainwater, well water, seawater, and water from snow melt. van Leeuwenhoek maintained those environmental samples in his house and observed that the types and relative concentrations of organisms present in his samples changed and fluctuated with respect to time. During the intervening centuries we have expanded our collective knowledge of these subjects which we now term to be environmental microbiology, but easily still recognize that many of the individual topics we have come to better understand and characterize initially were described by van Leeuwenhoek. van Leeuwenhoek was a draper by profession and fortunately for us his academic interests as a hobbyist went far beyond his professional challenges.

It is the goal of this series to present a broadly encompassing perspective regarding the principles of environmental microbiology and general microbial ecology. I am not sure whether Antonie van Leeuwenhoek could have foreseen where his discoveries have led, to the diversity of environmental microbiology subjects that we now study and the wealth of knowledge that we have accumulated. However, just as I always have enjoyed reading his account of environmental microorganisms, I feel that he would enjoy our efforts through this series to summarize what we have learned. I wonder, too, what the microbiologists of still future centuries would think of our efforts in comparison with those now unimaginable discoveries which they will have achieved. While we study the many

Christon J. Hurst in Heidelberg

wonders of microbiology, we also further our recognition that the microbes are our biological critics, and in the end they undoubtedly will have the final word regarding life on this planet.

Indebted with gratitude, I wish to thank the numerous scientists whose collaborative efforts will be creating this series and those giants in microbiology upon whose shoulders we have stood, for we could not accomplish this goal without the advantage that those giants have afforded us. The confidence and very positive encouragement of the editorial staff at Springer DE has been appreciated tremendously and it is through their help that my colleagues and I are able to present this book series to you, our audience.

Cincinnati, OH Christon J. Hurst

Volume Preface

Our goal as the authors of this book is to share a collective understanding that normally benign interspecies relationships do sometimes undergo changes whereby those relationships become detrimental.

Biologists have assigned a variety of definitions to each of the terms commensalism, symbiosis, and parasitism, with those definitions seeming to cross paths and disagree equally as do the biologists. We generally tend to view commensal relationships as being associations without inherent obligation, and for which there is no definable cost to any participant although some beneficial enticements can be involved. Symbionts are partners, by strict definition, with the organisms living together in a joint existence which seems more tightly involved and perhaps more mutually beneficial as compared to a commensal relationship. Symbiotic relationships often are so involved as to seem nearly obligatory for the participant species. But still, the principle assumption remains that both participants in a symbiotic association are deriving benefit from the relationship rather than receiving harm. In those cases where enticements are offered to favor the interspecies relationship, and often those enticements are nutritional, the term host may be used to help describe the major provider. Any energetic cost paid by the host to support presence of either commensal or symbiotic species presumably is outweighed by the beneficial and often protective nature of such relationships, with commensals and symbionts sometimes serving either to prevent or restrict the presence of other organisms that may be less favorably described as parasitic. Not all guests are welcome, and some initially may be considered benign but subsequently lose their welcome. The ecological definition of parasitism includes those less favorable situations that occur when a guest species obviously becomes deleterious. Thus, the dividing distinction between parasitism and these other types of interspecies relationships becomes a matter of detriment to the host.

Those microorganisms which normally might be considered either benign or even beneficial, but opportunistically become far more dangerous, very often are represented under the broad term ‘opportunistic pathogens’. However, rather than simply relying upon that term as a general cliché, the purpose of this book is helping to explain the current state of knowledge regarding conditions and mechanisms

which either allow or facilitate opportunistic pathogenicity. The trigger which allows that change can come in many ways. Sometimes, the effect results from a change in the host's capacity for mounting an effective immune response due to factors such as nutritional deprivation and coinfections. At other times, virus species either may have changed the opportunist or attacked the host's protective natural microflora. Even seemingly subtle environmental changes such as the amount of available sunlight, temperature, water and air quality parameters, can be enough to trigger dramatic shifts in delicately balanced interspecies relationships. The result of those shifts can be perceived as either a temporary bonanza for the pathogen or a disaster for the host. Knowledge regarding the nature of interactions which represent opportunistic pathogenicity in any single host–guest relationship valuably may then assist us towards unlocking the mystery of opportunistic pathogenicity for yet other systems.

We hope that you, our audience, will continue to carry forward the goal and purpose of this knowledge and of these efforts.

I am tremendously grateful to Hanna Hensler-Fritton, Andrea Schlitzberger, and Isabel Ullmann at Springer DE, for their help and constant encouragement which has enabled myself and the other authors to achieve publication of this collaborative project.

Cincinnati, OH Christon J. Hurst

Contents

Part I
Understanding Interspecies Relationships

Chapter 1
How Well Do Surrogate Hosts Serve as Model Systems for Understanding Pathogenicity

Christine Fink and Thomas Roeder

Abstract Experimental infection studies are of crucial importance to find and characterize virulence factors of pathogens or to identify novel compounds that can be used to treat the corresponding infections. The use of mammalian infection models including mice, rats, and guinea pigs is restricted due to several reasons including high costs, low statistical power, and ethical reservations. Simple, invertebrate models have been introduced as surrogate hosts as they are inexpensive, they can be used in great numbers, and doing experiments with them is not accompanied by ethical reservations. The soil nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster* have served as the most important surrogate hosts. Both organisms have served as workhorses in various biomedical disciplines. They combine simple and cheap handling and housing with an enormous armamentarium of genetic tools available to the scientific community. As their innate immune systems share substantial similarities with our own one, human bacterial and fungal pathogens often also infect these surrogate hosts. Nevertheless, it has to be kept in mind that both hosts share some drawbacks such as the apparent lack of adaptive immunity or the inability to survive at 37 °C. The latter point is relevant for especially those pathogens that require higher temperatures to become pathogenically triggered, and thus it would be helpful to seek the introduction of alternative models that can be used under these conditions. The greater wax moth *Galleria mellonella* exactly fits into this gap although it lacks most of the benefits supplied by the "classical" model organisms.

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1.1 Introduction

Experimental infection biology completely relies on having appropriate model systems to elucidate important factors relevant for the pathogen's capacity to colonize and damage the human or animal host. Ideally, these models should mirror the situation found in the customary host (in most cases the human host) as nearly as possible. Thus, mammalian models including mice, rats, rabbits, or guinea pigs that share much of our biology have served as model hosts for a huge variety of different bacterial and fungal pathogens. Although these models are characterized by a common set of advantages, their use in infection experiments is limited due to their huge biophysical complexity, exorbitant housing costs, and, most importantly, ethical reasons aiming to limit the use of mammals for animal experiments (Steinert et al. 2003). Thus, alternative models are required that might serve as valuable hosts, while excluding the drawbacks mentioned above.

One alternative approach that has gained some interest is the use of cell culture systems, ideally based on immortalized human cell lines that open the possibility to study at least some aspects of the pathogen's virulence and mechanisms of infection. Unfortunately, cell culture-based systems cannot represent the different levels of the complex interaction between host and pathogen. Thus, whole-animal-based infection systems are required to cover all major aspects relevant for this complex interaction of host and pathogen. Simple, nonvertebrate model organisms have come into the focus of experimental infection biology as they combine the advantages of cell culture systems (low costs, high ethical acceptance) with a whole-animal setting incorporating all major aspects of the infection process. Using invertebrate surrogate hosts became reasonable because the important findings of the last decades have revealed that the great majority of signaling pathways relevant for system development, including tissue homeostasis and innate immune responses, apparently evolved before vertebrates and invertebrates evolutionarily split. Thus, these important systems are conserved throughout the majority of the animal kingdom (Hemmrich et al. 2007; Salzet 2001). Moreover, those organisms that are genetically tractable open the opportunity to use yet that additional feature for mechanistic studies. This latter idea emanated almost 40 years ago, when the slime mold *Dictyostelium discoideum* was proposed to serve as a valuable model host (Depraitere and Darmon 1978). This simple, mostly unicellular organism has since been used to study the infection process and the virulence of intracellular bacteria such as *Legionella pneumophila* and *Listeria monocytogenes* (Steinert et al. 2003).

More recently, the use of more complex invertebrate models became popular, with the soil nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster* as their most important representatives. The major advantage relative to working with these two species is the unmatched wealth of information available for them combined with the easiness of their genetic manipulation, which is a prerequisite for understanding molecular infection mechanisms. Both organisms share a huge list of advantages including low-cost culturing, adaptability to high-

throughput systems, immune systems with similarities to our own innate immune system, fully sequenced genomes, and, most importantly, a plethora of available mutants and genetic tools allowing us to manipulate almost every gene in these model hosts. Moreover, both model organisms share a common lifestyle characterized by feeding on microorganisms such as bacteria and yeast. This natural way of confrontation with microorganisms predestines these models for infection experiments via the oral route. Although both organisms share this large list of pros, some cons have to be kept in mind. Neither worms nor flies are humans; they have a significantly different physiology and biology. Relevant for infection biology is that both seemingly lack any signs of adaptive immunity. Although this appears to represent an important drawback, most infections have to be managed, at least in the initial time of infection, by our innate immune system alone, and that result has its counterpart in these simple organisms. Moreover, the virulence of pathogens mainly relies on their ability to specifically interact with mucosal surfaces to reside there or to enter the body. The underlying processes involved with initiating infection appear to be well conserved from invertebrates to mammals (Steinert et al. 2003). Although using invertebrate surrogate hosts in experimental infection studies has been a success story, the limitations of the systems have always to be kept in mind. For instance, temperature-sensitive virulence factors that are operative only at the human body temperature of 37 °C could not be studied in the two model organisms *Drosophila melanogaster* and *Caenorhabditis elegans* as this would represent an abnormally elevated body temperature for those two invertebrate model organisms, and prolonged exposure of them to this elevated temperature is lethal. Nevertheless, pathogens adapted to this temperature can be studied in alternative models such as the greater wax moth *Galleria mellonella* that has been introduced into the field to exactly fill this gap (Lionakis 2011). Using one or more of these invertebrate hosts that tolerate higher temperature for infection studies offers not only the advantages mentioned above, but moreover it allows us to identify if a certain virulence factor is species specific or if it is relevant for a broad host range. In the following text, we want to focus on these three model hosts with respect to their recent and future contributions for the field of experimental infection biology.

1.2 *Caenorhabditis elegans*

Caenorhabditis elegans is a small free-living soil nematode that lives in temperate environments all over the world. Most individuals are hermaphrodites that self-fertilize. Only a small percentage of males can be found in natural populations. *Caenorhabditis elegans* had been introduced in 1974 by Sydney Brenner as a versatile model for developmental biology and neurobiology (Brenner 1974), an endeavor that was honored with the Nobel Prize for Physiology or Medicine in 2002 (Brenner 2003). *Caenorhabditis elegans* is small (1 mm) and translucent and can easily be cultivated on petri dishes using *Escherichia coli* or other bacteria as a food

source. These very simple growth conditions combined with the rapid generation time (2–3 days) and the incredibly high number of available mutants make this model ideally suited for a great number of study fields in biomedical research (Riddle et al. 1997). In addition, the ease of producing transgenic mutants or applying of RNAi targeted against endogenous target genes makes *C. elegans* ideally suited for experimental infection studies. Moreover, the extremely rich and versatile supportive resources available to all researchers make it easy to start working with this model (http://www.wormbook.org). Among the novel technical advantages that have become available in the most recent years is knowledge of the whole set of this species' genes as a functional study tool (Ashrafi et al. 2003; Poole et al. 2011; Yanos et al. 2012). Coming along in parallel with these technical breakthroughs, high-throughput approaches that can be used for infection studies or pharmacological studies have become popular (Burns et al. 2006; Okoli et al. 2009). In order to understand *C. elegans* as something more than a black box that is used in high-throughput screens, a basic understanding of its anatomy and immune system is expedient. Although *C. elegans* is a complex metazoan organism, it shows some peculiarities not shared by other invertebrate organisms. Nematodes are eutelic, meaning that adult worms have a constant number of cells that are slightly below 1000 for hermaphrodites and slightly greater than 1000 for males. Consequently, the organ composition of *C. elegans* is very simple. Most important for all aspects of the immune response is the gastrointestinal tract that encompasses a complex pharynx and a very simple intestine. As the lifestyle of *C. elegans* depends upon grazing on microorganisms, it is at constant risk of becoming infected via the oral route. Thus, it is especially well suited for all pathogens that usually infect humans via the oral route (Hilbi et al. 2007). To fight these potential pathogens that may be ingested by the nematode, a sophisticated, innate immune system is active (Irazoqui et al. 2010; Marsh and May 2012). It comprises signaling systems similar to the mammalian TLR-p38 MAPK and TGF-β pathways and an array of antimicrobial peptide compounds such as nlp-29 and cnc-2 (Pujol et al. 2008), as well as the large peptide family of so-called caenopores (Roeder et al. 2010; Irazoqui et al. 2010). Although substantial effort has been invested to elucidate the immune system of the nematode, some very important parts are still unknown, including the proteins that recognize bacterial or fungal components to trigger the above mentioned signaling cascades. For working with *C. elegans* as a surrogate host, one very big advantage is the possibility of using death as the important readout endpoint of an infection. In the following text, we will highlight only a very few examples of the numerous which have been published using *C. elegans* as a surrogate host of human bacterial and fungal pathogens (Table 1.1).

Table 1.1 Selected surrogate host models established in *Caenorhabditis elegans*

Microorganism	Inoculation method	References
Gram-negative bacteria		
Aeromonas hydrophila	Oral uptake	Couillault and Ewbank (2002)
Agrobacterium tumefaciens	Oral uptake	Couillault and Ewbank (2002)
Burkholderia cepacia	Oral uptake	Kothe et al. (2003)
Burkholderia pseudomallei	Oral uptake	O'Quinn et al. (2001)
Pseudomonas aeruginosa	Oral uptake, confrontation	Mahajan-Miklos et al. (1999); Tan et al. (1999b)
Salmonella typhimurium	Oral uptake	Aballay et al. (2000)
Serratia marcescens	Oral uptake	Mallo et al. (2002)
Yersinia spp.	Oral uptake	Darby et al. (2002)
Gram-positive bacteria		
Enterococcus faecalis	Oral uptake	Sifri et al. (2002)
Staphylococcus aureus	Oral uptake	Begun et al. (2005)
Streptococcus pyogenes	Confrontation	Jansen et al. (2002)
Fungi		
Histoplasma spp.	Oral uptake	Muhammed et al. (2012)
Candida albicans	Oral uptake	Okoli et al. (2009); Pukkila-Worley et al. (2011)
Cryptococcus spp.	Oral uptake	Mylonakis et al. (2002)

1.2.1 *Infection of* C. elegans *with Bacteria*

Caenorhabditis elegans was used as a surrogate host for a number of different human pathogens prior to the eventual identification of natural infection models for this species. Among the very few, naturally occurring bacterial pathogens that can infect the nematode in a natural environment, *Microbacterium nematophilum* is the best-studied example (Hodgkin et al. 2000; Gravato-Nobre et al. 2005). In contrast to the great variety of other pathogens that have been studied in *C. elegans*, *M. nematophilum* infects the anal region rather than attacking the intestine via the oral route. In the anal region, this microorganism induces a protective swelling response. Other naturally occurring pathogens including *Leucobacter* strains also have been identified that possess the potential to infect and kill the nematode in a natural setting (Hodgkin et al. 2013).

A huge list of potential bacterial pathogens encompassing both gram-positive and gram-negative species among which are intracellular bacteria has been studied in different *C. elegans* systems with the aim of understanding both virulence factors of the pathogen and mechanisms used by the host to fight these pathogens (Gravato-Nobre and Hodgkin 2005; Darby 2005). In the following, we want to focus on some pathogens in more detail including "major" human pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The latter of these was among the first

bacteria for which virulence mechanisms were studied utilizing *C. elegans*. *Pseudomonas aeruginosa* is a common bacterium found in water and soil. Although it is almost completely innocuous for healthy persons, immunocompromised patients are highly endangered. It can kill *C. elegans* by two completely different mechanisms, a fast killing caused by the secretion of toxins and a slow killing induced by conventional infection processes (Tan et al. 1999a, b). Studies using *P. aeruginosa* may have served a blueprint to utilize *C. elegans* as surrogate host. Researchers have in total tested more than 2000 bacterial mutants regarding their ability to kill *C. elegans*. Based on these studies, transcriptional activators such as LasR have been determined to be one of the quorum sensing systems that represent a major virulence factor (Tan et al. 1999a, b).

Staphylococcus aureus is frequently found on the human skin and within the respiratory tract. In most cases, this colonization is symptomless. The great number of antibiotic-resistant *S. aureus* strains causes huge problems in clinical settings. In *C. elegans*, *S. aureus* can cause a classical intestinal infection characterized by colonization of the intestinal lumen (Sifri et al. 2002). Screening efforts similar to those already described for *P. aeruginosa*, encompassing large mutant libraries, have enabled the identification of a large set of potential bacterial virulence factors (Bae et al. 2004; Begun et al. 2005).

A highly interesting example using infection caused by *Yersinia pestis*, the causative agent of bubonic plague, revealed microbial biofilm production as being a highly relevant virulence factor (Darby et al. 2002). The biofilm is formed around the mouth and subsequently that prevents feeding, which eventually leads to host death. Very similar to this biofilm formation is the situation found in the natural vector of *Yersinia*, which is the flea, where this biofilm formation is required for transmission (Darby et al. 2002).

1.2.2 Infection of C. elegans *by Fungi*

Nematodes have evolved a highly effective armamentarium of antifungal compounds. Upon fungal infection with *Drechmeria coniospora*, a diversified family of potential antifungal peptides is activated, which presumably offers the ability to fight a greater diversity of fungal pathogens (Pujol et al. 2008). Not only naturally occurring pathogens of *C. elegans* are able to trigger an immune response in the nematode following an infection, but also human pathogens such as *Candida albicans* hold this potential. Infection with *C. albicans* has been observed to induce characteristic sets of host genes that appeared to be yeast or fungus specific as very similar responses could be induced by confrontation with heat-killed yeast but not with bacterial pathogens (Pukkila-Worley et al. 2011). Based on comparable studies, a set of different assays has been developed that allow using *C. elegans* as a surrogate host for fungal infections. These include not only the conventional killing assay but also more sophisticated approaches such as the progeny permissive assay and the antifungal compound assay (Muhammed et al. 2012).

Thus, three major lines of assays have been used: those that focus on the fungal site utilizing panels of mutants, those that aim to identify novel antifungal components using infection models, and those that focus on the host site that aim to identify novel antifungal compounds (Anastassopoulou et al. 2011).

The human pathogens *Candida albicans* and *Candida neoformans* have been extensively used to infect *C. elegans* in different experimental settings, aiming to identify different virulence factors. Both fungi are able to establish a lethal infection in the intestine of *C. elegans*. Successful infection with these pathogens starts by breaking up yeast-infected hosts by means of a grinder to end up with a preparation of hyphal filaments, thus enabling the fungi to escape the body and proceed with infection of other animals. The subsequent appearance of filaments that have broken through the new host nematode's cuticle represents the endpoint of the infection process as this result always is accompanied by death of the host. In contrast to conventional life span assays, scoring these dead worms via the occurrence of fungal filaments is much easier, making it simple to adapt these screening systems to high throughput. Moreover, using temperature-sensitive mutants that are not able to produce any progeny above, e.g., 25 °C has the advantage that even longer infection periods can be scored quantitatively without being compromised by differing numbers of progeny.

A broad variety of assay techniques ranging in complexity from the very sophisticated to the very simple have been developed for screening antifungal compounds using a *C. albicans* infection. Green fluorescent protein-tagged pathogens are characterized by increasing green fluorescence in the intestine if an infection was successful. Compounds that reduce the fungal burden and therewith the green fluorescence in living nematodes can easily be identified using high-throughput screens. One of the various screens utilizing the *C. albicans*/*C. elegans* infection system led to the identification of novel antifungal compounds by Breger et al. (2007). In this comprehensive screen, two interesting compounds were revealed, namely, caffeic acid phenethyl ester, which is a component of the honey bee product propolis, and enoxacin, both of which also exhibit antifungal activities in murine infection models of candidiasis (Breger et al. 2007). This type of assay has been improved further to cope with greater numbers of compounds to be tested, which is a prerequisite to become a valuable model in screening assays for pharmacological companies. Both reduction of GFP signals within the animals in those assays that utilize GFP-tagged *C. albicans* and the occurrence of filamentous fungi indicative for killed worms in conventional assays are techniques ideally suited for high-throughput, quantitative approaches (Tampakakis et al. 2008).

The easiness of using these assays has enabled the study of more complex interactions, e.g., the interaction of *C. albicans* and *Acinetobacter baumannii* during a *C. elegans* infection. Surprisingly, coinfection with both microbes revealed a reduced virulence of *C. albicans* indicative for an interaction of *C. albicans* and *Acinetobacter baumannii* that inhibits important virulence factors of the fungus (Peleg et al. 2008). This is one showcase study that points to a complex interplay between *C. albicans* and *A. baumannii* that apparently is the consequence of reciprocal adaptation processes to suppress growth of the other

microbe. Thus, virulence factors of, e.g., *A. baumannii* that were identified in this screen hold the potential to represent interesting target molecules that interfere with the virulence of *C. albicans* for metazoan hosts (Peleg et al. 2008).

On the other hand, some *Candida* strains used for infection experiments are highly attenuated. This attenuation of virulence opens the possibility of identifying the underlying virulence factors. Those *C. albicans* strains that are defective in hyphenation including efgl deficiency and flo8 deficiency are much less virulent as compared to normal strains (Pukkila-Worley et al. 2009). Moreover, other sets of deficiencies have been identified, including some of which represent defectiveness in biofilm formation, and there are yet others whose deficient nature remains to be understood indicating that virulence of *C. albicans* for metazoan hosts is more complex than previously anticipated.

Besides *C. albicans*, *Cryptococcus neoformans* is the second fungal pathogen that has been studied in great detail utilizing *C. elegans* as a surrogate host infection model. Similarly as with *C. albicans*, the *C. neoformans* model is especially relevant for immunocompromised patients. *Cryptococcus neoformans* similarly is taken up orally by the host and establishes an intestinal infection. As already shown for a number of different human pathogens, yeast with higher virulence in murine models also shows a higher killing ability in the *C. elegans* assay. Especially noted is the finding that a component of the *C. neoformans* capsule is toxic to nematodes, as identified by the fact that heat-inactivated pathogens were still able to kill the nematodes (Mylonakis et al. 2002). In a larger screen utilizing several hundred *C. neoformans* insertion mutants, only very few were identified as having attenuated virulence. Of special interest is a mutation in the kin-1 gene that has shown reduced killing although no effect on colonization could be detected. This bipolar phenotype could be recapitulated in murine infection models (Mylonakis et al. 2004).

1.3 *Drosophila melanogaster*

The fruit fly *Drosophila melanogaster* has served as a workhorse for genetic studies since more than a century ago. It shares most of the same advantages for serving as a surrogate host in infection studies as noted above with the soil nematode *C. elegans*. The genome of *D. melanogaster* was sequenced more than a decade ago (Adams et al. 2000), and a plethora of mutants covering almost every gene is available (Ryder et al. 2007). Moreover, modern, *Drosophila*-specific, or *Drosophila*-adopted methods allow complex ways of genetic manipulation opening the opportunity to produce tailored fly models (Pfeiffer et al. 2008; Pfeiffer et al. 2010; Pandey and Nichols 2011). Most important in this context is the availability of bi- or tripartite expression control systems that allow for a tight spatial or even spatiotemporal expression control (Brand and Perrimon 1993; McGuire et al. 2003; Manning et al. 2012). Compared with *C. elegans*, *Drosophila* is characterized by an organ composition that more closely resembles the one seen

in mammals. This is comprised of a structured intestine, normal storage organs, and a body fluid system that is equipped with patrolling hemocytes. The fruit fly has served as the model for studying innate immunity since the rediscovery of this important aspect of its immune response repertoire almost two decades ago (Lemaitre et al. 1996). This "rediscovery" was initiated by the identification of Toll receptors serving as pattern recognition receptors of the innate immune system, which was honored by awarding of the Nobel Prize in Physiology or Medicine to Jules Hoffmann in 2011. Based on these early studies, it became apparent that *Drosophila* immunity not only serves as a blueprint for the mammalian innate immune system but that certain pathogens used the same strategies in flies and men to establish an infection in the host (Lemaitre and Hoffmann 2007; Ganesan et al. 2011; Hultmark 2003). The fly can react with a number of different responses toward the encounter with a pathogen. Exactly as in mammals, flies have potent humoral and local immune responses (Lemaitre and Hoffmann 2007; Hultmark 2003). While the major aim of the humoral immunity is to fight those pathogens that managed to invade into the body cavity, the local immune response aims to control the animals' surfaces, especially those in the intestine and the airways as they represent the most relevant entry points for pathogens. Protecting these mucosal surfaces is achieved by a multifaceted local immune system that is independent of Toll signaling. Instead, conventional innate immune responses that culminate in the production and release of antimicrobial peptides from these mucosal surfaces solely rely on IMD-signaling, a system that is homologous to our own TNF-a signaling system (Wagner et al. 2008, 2009; Tzou et al. 2000). This effective arm of the innate immune system is complemented by others controlling ROS production through the dual oxidase enzyme (Duox) (Ryu et al. 2010; Ha et al. 2005) and by danger signal-induced responses that are mediated through the transcription factor FoxO (Becker et al. 2010). These local, epithelial immune systems have not only the task to fight pathogens directly at the mucosal surfaces but also to shape the microbial community especially in the intestine to maintain a homeostatic situation between the host epithelia and the indigenous microbiota (Buchon et al. 2009) (Table 1.2).

For those pathogens that managed to penetrate these mucosal surfaces and that get access to the body cavity, the systemic immune system comes into play. It is composed of two major arms, the humoral and the cellular immune systems. The humoral arm of the systemic immune response reacts with release of antimicrobial compounds, namely, of antimicrobial peptides, into the hemolymph. Of central importance for this reaction is the fat body, the main immune-relevant organ in insects. This response can be triggered via two different signaling systems, the Toll and the IMD pathways. Whereas the Drosophila Toll pathway is homologous in all major components to the mammalian Toll-like signaling pathways, the IMD pathway is the insect counterpart of the mammalian TNF-a signaling system. Both signaling systems converge onto activation of NF-kB factors inducing transcription of relevant target genes. Besides these two pathways, others known from mammalian immune systems to be relevant, such as the JNK or the JAK/STAT pathways, are also involved in the fly's immune responses. The humoral immune system is

Table 1.2 Selected surrogate host models established in *Drosophila melanogaster*

Microorganism	Inoculation method	References
Gram-negative bacteria		
Burkholderia cepacia	Injection	Castonguay-Vanier et al. (2010)
Burkholderia thailandensis	Injection, oral uptake	Pilatova and Dionne (2012)
Pseudomonas aeruginosa	Injection	D'Argenio et al. (2001); Fauvarque et al. (2002)
Salmonella typhimurium	Injection	Shinzawa et al. (2009)
Serratia marcescens	Oral uptake	Cronin et al. (2009)
Yersinia spp.	S2 cells	Walker et al. (2013)
Gram-positive bacteria		
Enterococcus faecalis	Oral uptake	Teixeira et al. (2013)
Listeria monocytogenes	Injection, S2 cells	Cheng and Portnoy (2003); Ayres et al. (2008)
Staphylococcus aureus	Oral uptake, injection	Shiratsuchi et al. (2012)
Streptococcus pneumoniae	Injection	Chambers et al. (2012)
Fungi		
Aspergillus spp.	Injection, oral uptake, skin assay	Lionakis and Kontoyiannis (2010)
Fusarium spp.	Injection	Lamaris et al. (2007)
Scedosporium spp.	Injection	Lamaris et al. (2007)
Candida albicans	Injection, oral uptake	Glittenberg et al. (2011a, b)
Cryptococcus spp.	Oral uptake	Apidianakis et al. (2004)

supplemented by a highly effective cellular immune system. Three different types of hemocytes have been described in *Drosophila* that take different roles in the cellular immune response. Macrophage-like cells (plasmatocytes) ingest bacterial and fungal spores, while lamellocytes have the capacity to encapsulate and kill larger intruders. Crystal cells are a category of hemocytes that can release cytotoxic compounds and are involved in melanization (Lemaitre and Hoffmann 2007). Moreover, a potent, crystal cell-independent melanization cascade supplements the immune system at different levels. Taken together, the multiple layers of the fly's innate immune system are very similar to the defense systems of mammals that aim to inhibit colonization by potential pathogens.

Consequently and taking advantage of the various tools available, *Drosophila* has been used as a surrogate host for a number of different human pathogens with the aim to learn more about the infection mechanisms and the role of virulence factors. A number of different studies initially built the framework for later studies utilizing not just human pathogens but also insect- and invertebrate-specific pathogens including *Pseudomonas entomophila* and *Erwinia carotovora* (Liehl et al. 2006; Vodovar et al. 2005; Basset et al. 2000). Both of these pathogens are

able to infect flies via the oral route and have extensively been used to study the basic aspects characteristic for infections introduced via the oral route.

Based on these studies and the finding that the human and the fly's intestine shares a surprisingly high degree of similarities, those infection models utilizing oral infection procedures have become especially popular.

1.3.1 Drosophila *as a Surrogate Host for Bacterial Pathogens*

Principally, two types of infection are used. Although the "natural" oral infection that is achieved by mixing living bacteria to be tested with the fly food has several advantages, septic injury by pricking with needles that carry the bacteria into the body cavity often is also used. Infection with *Pseudomonas aeruginosa* was among the first attempts to use the fruit fly as a surrogate host, and this pathogen shows a strikingly broad host range covering not only mammals and invertebrates but also plants, making this potential pathogen ideally suited to be studied using surrogate hosts. In one of the pioneering studies in this field (D'Argenio et al. 2001), a panel of different *P. aeruginosa* mutants was used to assess their ability to kill the host within a certain time. They identified mutants defective in the gene switching ability involved in virulence factor regulation as being less effective in killing. In another study, type III secretion systems which inject effector proteins into host cells were found to be highly relevant for the severity of the infection (Fauvarque et al. 2002). Some clinical isolates (*P. aeruginosa* CHA as an example) of this bacterium are defective in the type III secretion system and as a consequence are less pathogenically effective in the *Drosophila* system. Moreover, the functionality of the quorum sensing system has been shown to be of great importance for enabling of a highly effective infection leading to quick host death (Chugani et al. 2001). More recent studies utilizing this infection model have revealed that biofilm formation during the infection process is highly relevant for virulence. During infection, a biofilm is formed in the crop, which is part of the digestive tract. Those strains defective in biofilm formation show not only a changed biofilm formation, triggering an immune response by the host, but they are also attenuated in the *Drosophila* infection model (Mulcahy et al. 2011).

Elucidating the infection mechanisms of *Serratia marcescens* has been tackled using different approaches (Nehme et al. 2007). Both injection by septic injury as well as oral infection with different *S. marcescens* isolates and clones have been performed and used to quantify the microbe's ability to kill the host. Septic injury with the *S. marcescens* isolate DB11 killed the host within the first day of infection. In contrast, lethality induced by the oral exposure route occurred after a few days of incubation within the host. Moreover, *S. marcescens* strains either deficient in O-antigen biosynthesis or characterized by reduced protease release show a reduced killing capacity, indicative for reduced virulence. On the host side, it became