**C. elegans**: model host and tool for antimicrobial drug discovery

Jonathan J. Ewbank¹,²,³,* and Olivier Zugasti¹,²,³

For almost four decades, the nematode *Caenorhabditis elegans* has been of great value in many fields of biological research. It is now used extensively in studies of microbial pathogenesis and innate immunity. The worm lacks an adaptive immune system and relies solely on its innate immune defences to cope with pathogen attack. Infectious microbes, many of which are of clinical interest, trigger specific mechanisms of innate immunity, and provoke the expression of antifungal or antibacterial polypeptides. In this review, we highlight some of these families of antimicrobial peptides (AMPs) and proteins that are candidates for the development of novel antibiotics. In addition, we describe how systems of *C. elegans* infection provide an increasing number of possibilities for large-scale in vivo screens for the discovery of new antimicrobial drugs. These systems open promising perspectives for innovative human therapies.

**Introduction: C. elegans as a model organism**

The nematode *Caenorhabditis elegans* is a genetically tractable multicellular organism that has been the subject of intense study for more than four decades. It has been successfully used as a model system to address fundamental questions in multiple fields of biology, including development, neurobiology and aging. In recent years, this tiny nematode worm has been applied to the study of microbial pathogenesis and host innate immunity, and used for drug discovery anddevelopment.

*C. elegans* is a self-fertilizing hermaphrodite with a rapid generation time. Each adult worm grows to a length of approximately 1 mm and under laboratory conditions can produce 300 genetically identical progeny in a 3-day life cycle. This allows the rapid expansion of strains and the establishment of large homogeneous populations. In the lab, *C. elegans* is propagated on agar plates or in liquid media with the auxotrophic *Escherichia coli* mutant strain OP50, and can live up to 3 weeks at room temperature. The entire genome sequence for *C. elegans* is available and many functional genomic approaches have been developed. Notably, RNA interference (RNAi) can be delivered systemically by feeding worms bacteria that express double-stranded RNA (dsRNA) targeting any gene of interest. Because dsRNA expression libraries covering almost 90% of the 20,000 genes in the *C. elegans* genome are available, many genome-wide RNAi screens have been performed (for a review, see Lamitina, 2006).

Furthermore, transgenic *C. elegans* strains can be readily created via microinjection of DNA (e.g. plasmids and/or PCR products), and its transparency renders the use of fluorescent reporter genes in vivo straightforward, as well as allowing direct real-time monitoring of infectious processes (Aballay et al., 2000; Labrousse et al., 2000).

There is an ever-growing list of Gram-positive, Gram-negative and fungal pathogens that are known to infect *C. elegans*, many of which are of clinical relevance (Darby, 2005; Sifri et al., 2005; Powell and Ausubel, 2008). A prominent example is the human opportunistic pathogen *Pseudomonas aeruginosa*, which was the first microorganism shown to be able to infect and kill *C. elegans*. In groundbreaking work, the Ausubel laboratory showed that many of the bacterial genes required for full virulence in the nematode were also important in other model systems (Tan et al., 1999a; Tan et al., 1999b). Coupled with the ease of culture and the possibility of automated handling, this has led to numerous in vivo large-scale screens for bacterial virulence factors, using *C. elegans* as a host (Kurz and Ewbank, 2007). In many cases, these studies have demonstrated that virulence factors involved in the killing of *C. elegans* are also required for pathogenesis in mammals. This opens up new avenues for the development of novel therapies that target specific virulence mechanisms.

Although *C. elegans* is unlikely ever to encounter many of the pathogens that are used in the laboratory in its natural environment of rotting fruit, *C. elegans* is continuously exposed to microorganisms, which can be either just a food source or also pathogenic. When confronted with pathogenic microorganisms, *C. elegans* activates protective mechanisms. These include an avoidance behaviour, triggered by the detection of specific microbial molecules, such as the cyclic pentadepsipeptide biosurfactant serrawettin W2, produced by some strains of *Serratia marcescens* (Pradel et al., 2007). When a pathogen cannot be avoided, *C. elegans* mounts an innate immune response, involving the activation of specific signalling pathways and leading to the production and release of defence molecules (Irazoqui et al., 2010). Among these immune effectors are a variety of antimicrobial peptides (AMPs) and proteins (Fig. 1). In the remainder of this Primer, we summarize what is known about these different families of antimicrobial factors, and discuss how *C. elegans* can be

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¹Centre d’Immunologie de Marseille-Luminy, Université de la Méditerranée, Case 906, 13288 Marseille cedex 9, France
²INSERM U631, 13288 Marseille, France
³CNRS UMR6102, 13288 Marseille, France
*Author for correspondence (ewbank@ciml.univ-mrs.fr)

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Antimicrobial proteins and peptides in C. elegans

The C. elegans genome encodes numerous candidate antimicrobial proteins and AMPs. Some were identified on the basis of sequence similarity with known antimicrobial factors in other species. Others were chosen for analysis because they are transcriptionally regulated upon infection. Many of these seem to be evolving rapidly and do not have equivalents even in other nematode species. Thus far, relatively few representatives of any family have been confirmed to have antimicrobial activity.

Caenopores

The first family described was the saposin-like proteins (SPPs; also known as caenopores), the nematode homologues of amoebapores (Banyai and Patthy, 1998; Leippe, 1999). It is a large family, with 28 different genes coding for at least 33 distinct proteins (Roeder et al., 2010). Caenopores share structural and functional features with amoebapores and with the cytotoxic vertebrate proteins granulysin and NK-lysin. In common with these proteins, they are pore-forming proteins that can kill bacteria (Roeder et al., 2010). They are predominantly expressed in the C. elegans intestine (Alper et al., 2007; Roeder et al., 2010) and their expression can be modulated by specific pathogenic bacteria (Alegado and Tan, 2008; Wong et al., 2007; Evans et al., 2008; Roeder et al., 2010). The caenopores are believed to act synergistically with other antimicrobial proteins, but their exact mode of action in vivo has yet to be fully characterized (Roeder et al., 2010).

Lysozymes and lectins

A second well-characterized class of antimicrobial proteins are the lysozymes, of which there are 15 in C. elegans (Schulenburg and Boehnisch, 2008). As is the case for the caenopores, their expression in the intestine can be induced by some pathogens (Mallo et al., 2002; Alper et al., 2007). It is thought that they are secreted into the intestinal lumen, where they act directly on microbes. Indeed, certain lysozymes have been shown to be important for the resistance of C. elegans to both Gram-positive and Gram-negative bacterial pathogens (Mallo et al., 2002; O’Rourke et al., 2006).

In addition, C. elegans has a very large family of lectins. They show complex patterns of gene regulation, with multiple lectin genes being upregulated in a non-overlapping manner in response to several different pathogens (Wong et al., 2007; O’Rourke et al., 2006; Troemel et al., 2006). They seem to be targets of multiple signal transduction pathways (Mallo et al., 2002; Alper et al., 2007) (O.Z., unpublished observations). In a few cases, their carbohydrate ligands have been defined (Nemoto-Sasaki et al., 2008). Some lectins

Fig. 1. C. elegans antimicrobial defences. (A) Basic anatomy of C. elegans. The worm is composed essentially of two concentric tubes – the epidermis (also called hypodermis) and the intestine – separated by a fluid cavity, the pseudocoelom. The physical barriers of the worm include a grinder, which mechanically disrupts the bacteria that form the worm’s normal diet, and the cuticle, which envelops the animal. (B) Selected AMPs and proteins in C. elegans. Invading microbes induce cell damage and are also believed to be detected via specific pathogen receptors. These two events trigger specific signalling pathways that lead to the production and release of a variety of specific antimicrobial proteins and AMPs (yellow boxes). Depending on the pathogen, these antimicrobial effectors are expressed specifically in a particular tissue, such as the pharynx, the intestine or the epidermis. Figure adapted with permission (Millet and Ewbank, 2004).
might therefore be involved in pathogen recognition (via binding to microbial surface sugars), others are possibly important for opsonizing microbes, whereas others seem to mask host epitopes that are the targets for specific microbial effectors (Schulenburg et al., 2008; Ideo et al., 2009).

ABF peptides

The identification through biochemical means of the Ascaris suum antibacterial factor (ASABF) peptides in the parasitic nematode Ascaris suum allowed the annotation of six homologous antibacterial factor (ABF) peptides in C. elegans. These peptides are somewhat similar to vertebrate defensins, and recombinant ABF-2 has a demonstrated broad-spectrum antimicrobial activity in vitro, with the greatest effect observed on Gram-positive bacteria (Kato et al., 2002). Based on its sequence, ABF-2 is thought to kill microbes by membrane disruption (Zhang et al., 2000). Even in the absence of immune challenge, ABF-1, ABF-2 and ABF-3 are constitutively expressed. ABF-1 and ABF-2 are mainly expressed in the pharyngeal tissues, whereas ABF-1 and ABF-3 are also detected in the intestine (Kato et al., 2002; Alper et al., 2007). Because, in decomposing fruit, C. elegans is constantly exposed to potential pathogenic microorganisms, the constitutive expression of these ABF peptides could represent a preventative mechanism of defence, or be part of the C. elegans digestive machinery. It has also been shown that expression of the ABF peptides is upregulated following infection by different pathogens. Upon infection by the human pathogen Salmonella typhimurium, the expression of ABF-2 is greatly increased, and this acts to limit bacterial proliferation in the intestinal lumen (Alegado and Tan, 2008). In addition, infection of C. elegans by Cryptococcus neoformans leads to the upregulation of ABF-1 and ABF-2 expression (Means et al., 2009), whereas exposure to Staphylococcus aureus is followed by a weak expression of ABF-3 (Alper et al., 2007). Hence, ABF peptides seem to play a role in C. elegans immune response to a diverse group of pathogens.

NLPs and CNCs

Just as the mammalian intestine is constantly in contact with potential pathogens, so is the epidermis of C. elegans. There are innumerable bacterial and fungal pathogens that attack nematodes following an initial attachment to the cuticle. Infection by the Gram-positive bacterium Microbacterium nematophilum causes a protective swelling response (Hodgkin et al., 2000; Nicholas and Hodgkin, 2004), whereas the fungal pathogen Drechmeria coniospora triggers an epidermal immune response characterized by the expression of antimicrobial genes encoding neuropeptide-like peptides (NLPs) and a family of closely related peptides named caenacins (CNCs) (Couillault et al., 2004; Pujol et al., 2008; Zugasti and Ewbank, 2009). Recombinant NLP-31 was shown to have an antifungal activity in vitro against D. coniospora, as well as a potent effect against several other microbes, including the human opportunistic pathogen Aspergillus fumigatus (Couillault et al., 2004).

Most of the infection-inducible genes encoding NLPs and CNCs are grouped in two genomic clusters. The nlp-29 cluster comprises nlp-27 to nlp-31 and the adjacent nlp-34 gene, whereas the cnc-2 cluster groups together the genes cnc-1 to cnc-5, and cnc-11. Overexpression of the genes of the nlp-29 or cnc-2 clusters enhances resistance to D. coniospora infection, demonstrating that these genes can contribute in vivo to host defence (Pujol et al., 2008; Zugasti and Ewbank, 2009). Several NLP- and CNC-encoding genes, including nlp-29 and cnc-4, are also induced by infection by the nematode-trapping fungus Monacrosporium haptotylum, which, similar to D. coniospora, infects via the cuticle (Fekete et al., 2008). Contrary to initial reports, however, none of the genes encoding NLPs or CNCs is induced by S. marcescens (Wong et al., 2007; Zugasti and Ewbank, 2009). They are also not induced by M. nematophilum nor P. aeruginosa (O’Rourke et al., 2006; Shapira et al., 2006). By contrast, the expression of nlp-29 is upregulated by the bacterium Leucoobacter chromireducens, which establishes a uterine infection (Muir and Tan, 2008). This suggests that both the nature of the pathogen and the route of infection contribute to the induction of specific defence genes in C. elegans.

Microarray studies have shown that, in addition to induction of the NLP- and CNC-encoding genes, D. coniospora infection also leads to the expression of numerous genes that potentially encode AMPs. These include families such as the fungus-induced proteins (FIPs), FIP-related proteins (FIPRs) and glycine-rich secreted proteins (GRSPs) (Pujol et al., 2008). Indeed, transcriptome analyses of the response of C. elegans to infection have been a rich source of leads for those looking for natural antibiotics.

C. elegans as a tool for therapeutic drug discovery and development

The growing spread of multi-drug-resistant bacteria, as exemplified by carbapenem-resistant Enterobacteriaceae carrying metallo-β-lactamase-1 (NDM-1; an enzyme that confers resistance to a broad range of β-lactam antibiotics), highlights the urgency to find new antimicrobial compounds (Bush, 2010). Traditional in vitro assays measuring growth inhibition or killing of pathogens, as well as whole-cell drug screens, are not likely to meet the expected future needs of drug development. In light of the properties outlined above, C. elegans is a very convenient whole organism model in which to identify or assay antimicrobial compounds.

One promising approach is to find compounds that interfere with bacterial quorum sensing mechanisms, because these can influence the expression of virulence genes. As a proof of principle for this approach, a potent antagonist of quorum sensing in the bacteria Chromobacterium violaceum was shown to protect C. elegans from bacterial killing (Swem et al., 2009). This validates the idea that compounds that block quorum sensing could be identified using C. elegans and potentially developed as antimicrobial drugs.

Using a different approach, the antibiotic action of membrane-active cationic AMPs (CAMPs) from frog skin was recently evaluated in C. elegans. Esculetin and temporin, two CAMPs that are candidates for drug development, were found to be highly efficacious in promoting C. elegans survival following infection by P. aeruginosa. For esculetin, its effect was shown to be due to permeabilization of the membrane of the pathogen within the infected nematode (Uccellletti et al., 2010). But, in addition to enabling the identification of microcidal or microstatic molecules, the use of C. elegans also allows detection of molecules that enhance immune defences in vivo. Indeed, precisely this sort of drug candidate has been found in compound library screens that assay the effect of drugs on C. elegans survival following infection. In the first screen of this type, a liquid-based assay in 96-well microtiter plates was developed to test the potential of thousands
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of synthetic compounds and natural extracts to cure C. elegans following Enterococcus faecalis infection (Moy et al., 2006). In this screen, 16 compounds and nine extracts were shown to promote C. elegans survival (0.25% and 0.8% hit rates, respectively). Among them, several products had essentially no effect on bacterial growth in vitro, so would not have been identified in more conventional screens (Moy et al., 2006). Since this pioneering work was published, the assay has been improved, miniaturized and fully automated. An important improvement involved the application of a fluorescent dye that allowed the researchers to discriminate between live and dead worms (Fig. 2). With this newer high-throughput screen, close to 40,000 compounds and extracts were tested, allowing the identification of 28 novel antimicrobials (Moy et al., 2009). Again, in contrast to traditional antibiotics, the in vivo effective dose of many of the compounds identified was significantly lower than the minimum inhibitory concentration (MIC) needed to prevent the growth of E. faecalis in vitro (Moy et al., 2009).

Similar high-throughput in vivo assays have been used to screen for antimicrobials that are effective against other human pathogens, including the fungus Candida albicans. A first screen of 1266 compounds with known pharmaceutical activities identified 15 that prolonged survival of nematodes infected with C. albicans and inhibited in vivo filamentation of the fungus, which is a key step in Candida pathogenesis in mammals. Among the compounds identified in the screen, caffeic acid phenethyl ester and the fluoroquinolone agent enoxacin also exhibited marked antifungal activity in a mouse model of candidiasis, demonstrating the relevance of these approaches (Breger et al., 2007). A second, improved screen was recently conducted, bringing the total number of different tested compounds to close to 4500. A further 19 compounds conferring an increased survival after infection of C. elegans by C. albicans were identified. The robustness of this screen was illustrated by the detection of essentially all of the known antifungal agents within the chemical library. In addition to these seven clinically used antifungal drugs, the screen revealed an antifungal activity for 12 new compounds, including three immunosuppressive drugs (Okoli et al., 2009). These assays also provided the opportunity to assess the relative MIC and the effective concentration in vivo, as well as the toxicity of these compounds, in a single assay.

Conclusion

In summary, C. elegans produces a wide variety of potential antimicrobial proteins and peptides. Many of these have no structural equivalents in other species, and their actual spectrum of activities remains to be tested. Given the hostile environment that C. elegans lives in, it is likely that many of them have potent antimicrobial activities and could be used as a starting point for the development of new antimicrobial drugs. There are, however, many barriers to bringing oral peptide-based antibiotics from any species to the market. These include issues of drug delivery, pharmacokinetics and immunogenicity. The development of Cytolex, derived from a magainin AMP from frogs, for the treatment of infection in diabetic foot ulcers suggests, however, that there is much promise for AMPs as topical antibiotics. The nematode could be a rich source of potential leads for the future development of such drugs.

C. elegans has already established itself as a powerful tool to screen for antimicrobial activity in compound libraries. For many reasons, this nematode cannot completely replace mammalian models for drug discovery and development, and its relevance to human health is clearly limited given the evolutionary distance and obvious physiological differences that separates man from worm. Among these, the fact that C. elegans cannot be cultured at 37°C restricts the range of pathogens that can be studied with the nematode. Additionally, C. elegans has a very efficient detoxification system, which relies in part on an extremely rapid defecation cycle and on its large complement of cytochrome P450 genes. This limits the capacity to identify compounds that act via modulation of host defences.

Nevertheless, C. elegans has many practical advantages, is amenable to rapid, low-cost, large-scale in vivo screens and does not raise any of the ethical concerns associated with the use of monkeys, or even mice, for drug testing. With the ever-decreasing cost of DNA sequencing, C. elegans also offers an unparalleled opportunity to study drug action via genetic screens (Doitsidou et al., 2010) or transcriptome studies using RNA-seq (Hillier et al., 2009). In the future, it is likely that C. elegans will be increasingly used to dissect the virulence of a pathogen, to find.

Fig. 2. A simplified protocol to screen in vivo for new antimicrobial compounds using an established C. elegans infection system and an automated high-throughput assay. After culture and amplification of nematodes on growth plates (seeded with a non-pathogenic E. coli strain), synchronized populations of worms are transferred to infection plates that have been seeded with pathogenic E. faecalis. After half a day, worms are washed off the plates and 15-25 individuals are added to each well of 96-well (shown here) or 384-well microtiter plates that have been pre-loaded with different compounds. After 5 days, the worms in each well are assayed for their survival, using the vital dye SYTOX. Because it is only taken up by dead worms, measuring worm survival can be automated using image analysis software. Wells in which worms live substantially longer contain candidate antibiotics. The figure summarizes the approach taken by Moy et al. (Moy et al., 2009).

Practical advantages of C. elegans as a model system for drug development

• Simplicity and low cost of culture.
• Targeted by many virulence factors of human pathogens.
• Whole-animal model.
• Suitable for rapid high-throughput screening in vivo.
• Permissive legislation: none of the ethical concerns associated with higher animals.

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leads for antimicrobial drug discovery, and to untangle the molecular mechanisms that control resistance and susceptibility to disease.

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