Combating Antibiotic Resistance: Identification of Compounds Targeting Bacterial Virulence and Host Innate Immunity

PhD Thesis

Henrik Jakobsen

Department of Science, Systems and Models, Roskilde University

Department of Microbiology and Infection Control, Statens Serum Institut

Supervisors:

Professor Anders Løbner-Olesen

Professor Karen A. Krogfelt

Senior Scientist Carsten Struve

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Abstract

The problem of antibiotic resistance among pathogenic bacteria is as old as antibiotics themselves. The antibacterial drugs in use today all, with few exceptions, make use of targets such as: cell wall synthesis, DNA replication, RNA transcription, protein synthesis and folic acid synthesis. Common for all of these drugs is that resistant bacteria have emerged only a few years after their introduction into clinical use. By continuously discovering new classes of antibiotics and altering the old ones, we have managed to stay one step ahead of the bacteria. However, the last few decades have seen a rapid decline of new antibiotics in the clinical pipeline.

In this thesis I describe a number of attempts to identify new types of compounds against pathogenic bacteria. These compounds were sought to target the virulence regulation of the pathogens or the innate immune response of the host.

I describe our attempt to identify small circular peptide inhibitors of the virulence regulator protein ToxT, from pathogenic *Vibrio cholerae*. We designed a screening system for selecting inhibitors of ToxTs function as a transcriptional activator of the Cholera toxin. We used this screening system to screen a library of approximately 500.000 small (9 amino acids) circular peptides. However, this screen did not reveal any inhibitors.

We also show that the RNA chaperone Hfq is required for full virulence of pathogenic *Escherichia coli* in a *Caenorhabditis elegans* infection model. *hfq* mutants were also more sensitive to oxidative stress from hydrogen peroxide in an *in vitro* assay. On the basis of these finding we employed a 2-hybrid screen for protein-protein interaction between Hfq monomers. Using this system we performed a screen of two smaller compound libraries (the 1280 pharmacologically active compounds $LOPAC_{1280}$ library and the MicroSource: FDA-approved US drug collection, comprised of 1040 compounds). We identified one possible candidate, nordihydroguaiaretic acid (NDGA).

This compound was tested in the *C. elegans* infection model. We found some efficacy, but much less than seen in the corresponding *hfq* mutant *E. coli* strain.

Lastly, we show that the alkaloid compound Harmane (2-methyl-β-carboline) acts as an immuno-modulating drug in *C. elegans* resulting in a significant increase in the lifespan of nematodes infected with a number of human pathogens. We show that Harmane stimulates the innate immune response of the nematode and that this seem to help the nematode tolerate stress of the infection, rather than to directly combat the pathogens. Since the innate immunity of *C. elegans* has a high degree of evolutionary conservation, drugs such as Harmane could be possible alternatives to classic antibiotics. The *C. elegans* model could prove to be useful for selection and development of immuno-modulating drugs.

Resumé

Problemerne vedrørende antibiotikaresistens blandt patogene bakterier er lige så gammelt som vores brug af antibiotika. De antibakterielle stoffer som anvendes i dag er, med få undtagelser, alle rettet mod bakterielle funktioner, så som: bakteriernes cellevæg, DNA replikation, RNA transskription, protein syntese og folinsyre syntesen. Fælles for alle disse antibakterielle stoffer er, at resistente bakterier er fremkommet blot få år efter påbegyndelse af brugen af stofferne til behandling. Ved hele tiden at finde nye antibiotikaklasser og ved løbende at ændre og optimere de allerede kendte stoffer, har vi formået at holde os ét skridt foran bakterierne. Men, inden for de seneste 30 år har vi været vidne til at stadig færre nye stoffer finder vej fra industrien og ud på markedet.

I denne afhandling beskriver jeg at antal forsøg på, at identificere nye stoffer mod patogene bakterier. Disse stoffer er søgt rette imod reguleringen af virulens i bakterier eller de søger at stimulere det medfødte immunsystem i værten.

Jeg beskriver vores forsøg på at identificere små cirkulære peptider rette imod det virulensregulerende protein ToxT fra *Vibrio cholerae*. Vi har designet et screeningsystem til at selektere inhibitorer af ToxTs funktion som transskriptionel aktivator af kolera toksinet. Vi anvendte screeningsystemet til at gennemsøge et bibliotek med cirka 500,000 små (9 aminosyrer) cirkulære peptider. Desværre afslørede vores forsøg ikke nogen inhibitorer.

Vi viser også at RNA chaparonen Hfq er nødvendig for at opnå fuld virulens i patogene *Escherichia coli* i en *Caenorhabditis elegans* infektionsmodel. Hfq mutanter viste sig også at være mere udsatte overfor oxidativ stress, i form af hydrogenperoxid, i et *in vitro* forsøg. På basis af disse resultater valgte vi at anvende et 2-hybrid screen for at undersøge protein-protein interaktioner imellem Hfq monomerer. Ved hjælp af dette assay gennemsøgte vi to mindre stofbiblioteker (de 1280 farmakologisk aktive stoffer i $LOPAC_{1280}$ bibliotket, samt MicroSource: FDA-approved US drug collection, med 1040 stoffer). Vi identificerede én mulig kandidat, nordihydroguaiaretic acid (NDGA). Vi testede dette stof i *C. elegans* infektionsmodellen. Vi kunne vise nogen effect, men ikke svarende til effekten af *hfq* mutationen i den samme *E. coli* stamme.

Til sidst viser vi at alkaloidet, Harmane (2-methyl-β-carboline) er i stand til at modulere det innate immunsystem i *C. elegans*, med det resultat at ormene lever signifikant længere, når de er inficeret med en række forskellige patogene bakterier. Vi viser at Harmane stimulerer det innate immunsystem i nematoden og at denne stimulation

tilsyneladende gør ormen bedre i stand til at tolerere den stress der følger af infektionen, snarere end direkte at bekæmpe infektionen. Da det innate immunsystem i *C. elegans* i høj grad er evolutionært bevaret, kan man formode at stoffer som Harmane vil kunne anvendes som et alternativ til antibiotika. *C. elegans* infektionsmodellen kunne vise sig nyttig til identifikation og udvikling af sådanne immunstimulerende stoffer.

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Introduction

Since their introduction into clinical use more than half a century ago, antibiotics have been immensely successful. This is despite the fact that shortly after their introduction into widespread use, bacteria, resistant to virtually every drug, began to emerge (as outlined in figure 1). Sulphonamides were the first large-scale clinically successful antibacterial drugs. They were introduced in the mid-1930s and by the end of that decade sulphonamide-resistant *Streptococcus pyogenes* had emerged. Penicillin-resistant *Staphylococcus aureus* were detected in hospitals in the 1940s, the same decade that penicillin was introduced (Levy and Marshall 2004). During the 1940s and 1950s these single-drug resistance problems were overcome by a wave of novel classes of antibiotics such as cephalosporins, glycopeptides and tetracyclines. In the late 1950s and early 1960s multidrug resistant (MDR) strains started to emerge. In Japan, strains of *Shigella* were isolated from patients suffering from dysentery. These strains were resistant to several drugs, including sulphonamides, streptomycin, chloramphenicol and tetracycline (Davies 1995). It was also discovered that most of the patients carrying MDR *Shigella* strains also had MDR *Escherichia coli* in their intestines.

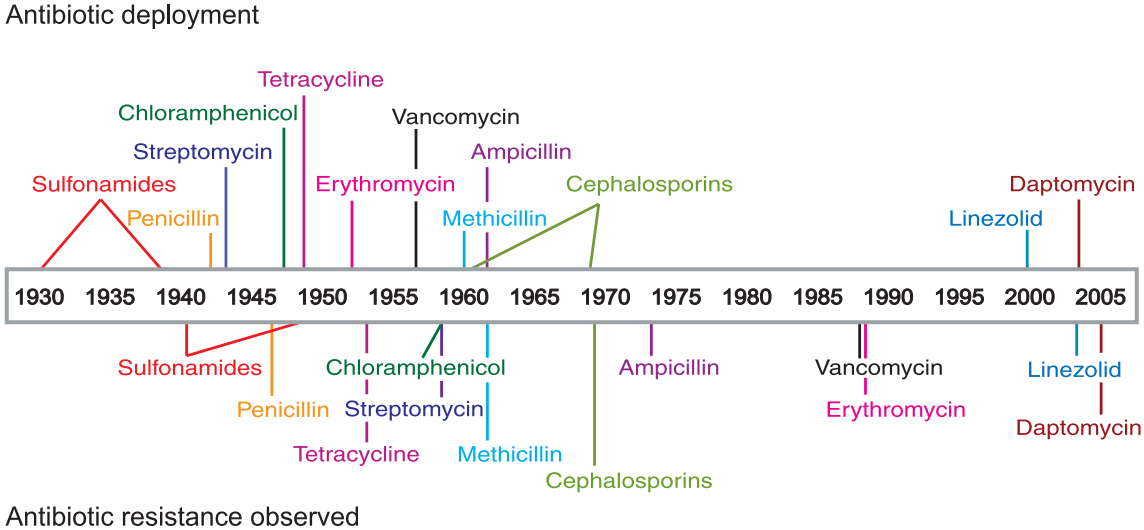

Figure 1. Timeline showing the year when different antibiotics were introduced into clinical use, as well as the year when bacteria resistant to the drugs were discovered among patients. Figure adapted from (Clatworthy *et al.* 2007).

This indicated for the first time that drug resistance genes could be transferred between bacteria and not only of the same species (Davies 1995, Franklin *et al.* 2005). Today MDR strains of *Mycobacterium tuberculosis, Enterococcus faecium, Klebsiella pneumoniae, Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus* (MRSA) are a common health problem, especially in developing countries. These MDR strains make treatment considerably more costly and sometimes unsuccessful (French 2010, Levy and Marshall 2004).

The driving force behind the increasing drug resistance is undoubtedly the immense amounts of antibacterial drugs used in the prophylaxis and treatment of humans and animals worldwide (Austin *et al.* 1999, Malhotra-Kumar *et al.* 2007). Efforts clearly have to be taken in order to minimize the unnecessary use of antibacterial drugs, in order to control the spread of resistance. However, a renewed search for new antibacterial drugs and new targets for drugs is also necessary. Despite the obvious need for new and improved treatments, only one truly new class of antibacterial drugs, the oxazolidinones, has come into clinical use within the last three decades (Shinabarger *et al.* 1997). A member of this class, Linezolid, has found clinical use against MRSA; however, strains resistant to the drug have already been reported (Tsiodras *et al.* 2001).

The most popular strategy for circumventing the resistance problems with existing drugs has been to design new drug-analogous. A number of new types of aminoglycoside and tetracycline drugs have been developed in this fashion (Theuretzbacher 2012). Another strategy to extend the lifespan of existing drug has been to develop inhibitors targeting the resistance mechanisms. An example of such inhibitors is the β-lactamase inhibitor avibactam (Ehmann *et al.* 2012). It is currently being tested in clinical trials in combination with the β-lactamase-sensitive cephalosporin analogues ceftaroline and ceftazidime. The hope is that such inhibitor can counter the growing problem of extended-spectrum β-lactamases (ESBLs) produced by *Escherichia coli* and *Klebsiella* strains (French 2010).

The problem of antibiotic resistance is unavoidable when using antibiotics that target essential mechanisms of bacterial growth, since it is a result of simple evolution of the bacteria themselves. In the following sections I will give a short description of classical antibiotics and how they work, as well as the resistance-strategies that bacteria utilise to survive them. I will also give an introduction to some of the new strategies for developing new antimicrobial drugs that might circumvent this challenge, namely anti-virulence drugs and immune-stimulatory drugs.

Antibacterial drugs

The general strategy when combating bacterial infections is to kill or inhibit the growth of the bacteria without harming the patient. In order to achieve this, selective toxic drugs are used. The antibacterial drugs target essential features in prokaryotic organisms that are absent or different in eukaryotes. The vast majority of antibacterial drugs inhibit either the bacterial cell wall synthesis or bacterial protein synthesis, but other processes are targeted as well. Figure 2 gives an overview of some common targets and examples of drug classes and drugs targeting these.

Inhibitors of bacterial cell wall synthesis

The prime target of most antibacterial drugs is the bacterial cell wall. Practically all bacteria have a cell wall, with a few exceptions such as mycoplasmas, whereas eukaryotic cells lack this feature. Most eukaryotic animal cells exist in a controlled environment with constant temperature and osmolarity. This allows the cells to survive with only the soft cell membrane as an outer barrier.

Bacteria, on the other hand often exist in hostile environments with changing temperatures and osmolarity. This means that bacteria need a stronger and more protective layer outside the cell membrane. This layer is the cell wall; it protects the cell from the environment and preserves the shape of the bacterial cell. Penicillins and cephalosporins belong to the class of antibiotics known as β-Lactams, since they both contain a β-lactam ring.

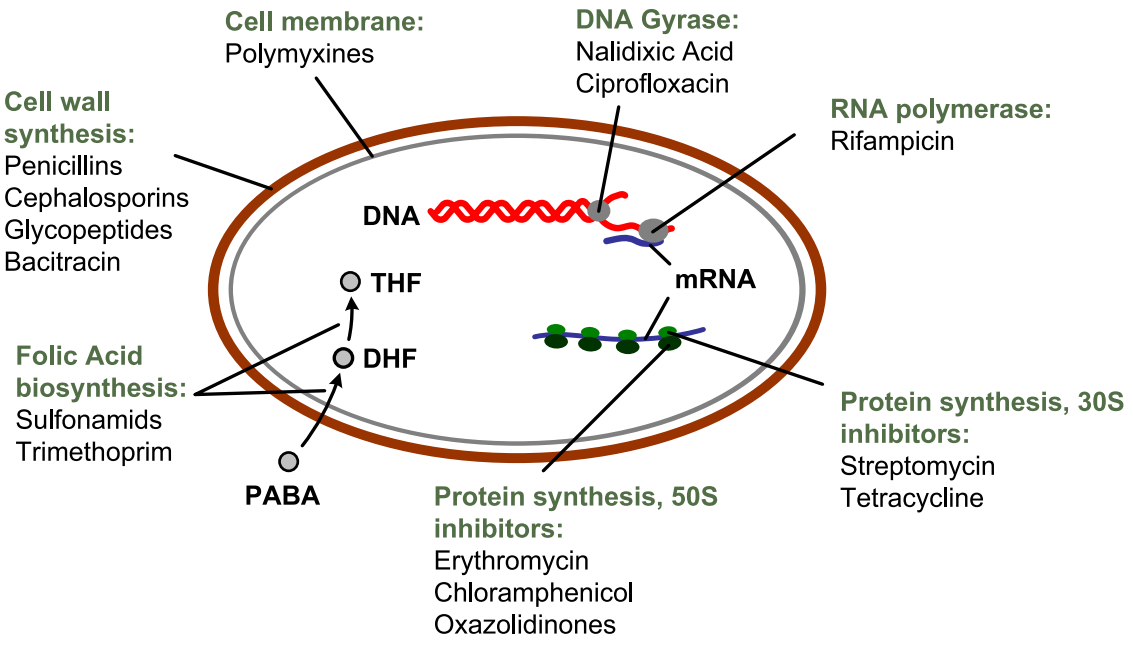

Figure 2. An overview of different antibacterial drugs and their targets in the prokaryotic cell. THF, tetrahydrofolate; DHF, dihydrofolate; PABA, para-aminobenzoic acid. The figure was adapted from (Alberts 2008).

Different types of Penicillins and cephalo-sporins can be produced by exchanging the side-chains of the molecules. This has broadened their spectrum and combated resistance problems. β-Lactams work by interfering with the cross-linking of the peptidoglycan layer. They do this by binding to the various Penicillin-Binding Proteins (PBPs) involved in biosynthesis of the peptidoglycan layer (Franklin, Snow and Franklin 2005, Tomasz 1979). This results in weakening of the peptidoglycan layer which eventually triggers lysis of the cell.

Inhibitors of bacterial protein synthesis

Many clinically important antibacterial drugs inhibit bacterial protein synthesis by binding with the prokaryotic 70S ribosome, without affecting the 80S eukaryotic ribosome. However, the mitochondrial ribosome bares great resemblance to the prokaryotic ribosome, probably because mitochondria have derived from endo-symbiotic bacteria during the course of evolution. This means that a safe inhibitor of bacterial protein synthesis must be able to gain access to bacterial ribosomes, but not mitochondrial.

Inhibitors of protein synthesis include natural antibiotics isolated from actinomycete, such as tetracycline (isolated from *Streptomyces aureofaciens*) and chloramphenicol (from *Streptomyces venequelae*), as well as the aminoglycoside antibiotic streptomycin from *Streptomyces griseus*. The oxazolidinones are synthetic antibacterial drugs. As mentioned earlier they are the only true new class of antibacterial drugs to be discovered in the last 30 years.

Drugs that inhibit the protein synthesis can be divided into two major classes: 50S inhibitors and 30S inhibitors. 50S ribosome inhibitors include macrolides (such as erythromycin), amphenicols (for example chloramphenicol) and oxazolidinones (for example linezolid). 30S inhibitors include tetracyclines and aminoglycosides (for example streptomycin). Common to these drugs is that they bind to components of the ribosome and physically block its function (Patel *et al.* 2001). This can be either initiation or elongation of the protein, or in the case of aminoglycosides, promotion of tRNA mismatching during elongation – resulting in mistranslation of the mRNA (Davis 1987).

Inhibitors of nucleic acid biosynthesis

The synthesis of DNA and the different types of RNA is essential to the growth of bacteria. Antibacterial drugs can either interfere with the synthesis of the building blocks of DNA and RNA, or they can target the enzymes involved in DNA or RNA synthesis.

Quinolones such as nalidixic acid and the newer ciproflaxin, are among the very few inhibitors of the DNA replication. More precisely, they act by targeting the bacterial topoisomerases. The DNA of a bacterial cell is supercoiled in order to minimize its size. The enzyme that introduces negative supercoiling is named type II topoisomerase or DNA gyrase. The supercoiling process involves the introduction of double stranded nicks in the DNA; the quinolones inhibit the religation of the nicks, and accumulation of these double stranded nicks in the genome eventually kills the bacterium (Drlica *et al.* 2008). The early quinolones such as nalidixic acid were only effective against Gram-

negative bacteria, but newer compounds such as ciprofloxacin are also effective against Gram-positive bacteria.

In the sections above we have seen how antibiotics bind to key components in the bacterial cell and thereby disrupt essential life processes. This view on the killing mechanisms of antibiotics could be called the *classical view*. In recent years a more *modern view* has emerged. Not in place of the *classical view*, but rather in addition to it (Kohanski *et al.* 2010). In this view, it is proposed that treatment with bactericidal antibiotics leads to production of harmful hydroxyl radicals inside the bacteria. These oxidative species inflict damage to DNA, lipids and proteins and thereby contribute to the killing of the bacteria (Kohanski, Dwyer and Collins 2010, Kohanski *et al.* 2007).

Antibacterial drug resistance

Bacteria and antibiotic producing microorganisms have existed side by side up through evolution, so it is not surprising that bacteria have evolved defences against antibiotics.

Intrinsic resistance

Some bacteria are naturally resistant to certain antibacterial drugs. Mycoplasmas lack the cell wall and are therefore unaffected by β-lactamases and other antibacterial drugs targeting the cell wall synthesis. The Gram-positive mycobacteria have a dense lipid coat in the outer layers of the cell wall; this layer resembles the outer membrane of Gram-negative bacteria and is a barrier against many common antibacterial drugs (Nikaido 1994). Mycobacteria also exhibit a very slow growth rate; treatment of infections, such as tuberculosis and leprosy, therefore requires several months of continuous treatment.

The complex outer membrane of Gram-negative bacteria is more rigid than the plasma membrane and less penetrable by many antibiotics. The drugs therefore rely on the porin channels for their entry into the periplasmic space. The opportunistic pathogen

Pseudomonas aeruginosa is characterized by having very restricted porin channels making it resistant to many antibacterials (Yoneyama *et al.* 1995).

Many bacteria (including *P. aeruginosa*) can in some situations form biofilm. It is believed that cells living in biofilm change their growth dynamic, rendering them unsusceptible to many antibacterial drugs. Intrinsic resistance is fortunately often predictable and infections can be treated by choosing the right type of drug. A bigger challenge is posed by the much more unpredictable phenomenon of acquired resistance.

Acquired resistance

The emergence of resistant bacteria among species that would normally be considered susceptible has made it clear that bacteria can acquire resistance. Many different biological mechanisms behind acquired resistance have been characterized. Figure 3 illustrates some of the most common mechanisms. Various mechanisms can provide bacteria with resistance towards antibiotics; the most common are: target-site modification, antibiotic inactivation and drug efflux pumps.

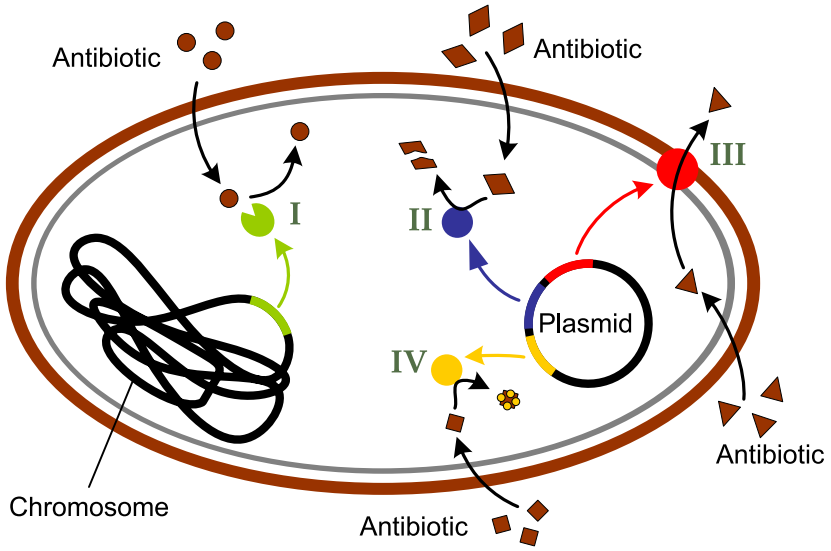

Figure 3. A variety of resistance mechanisms have evolved among bacteria. I: genetic mutations can alter the binding site of the antibiotic and prevent binding. II: enzymes can inactivate the drugs, such as β-lactamases that destroy penicillins and cephalosporins. III: Efflux pumps can actively pump the antibiotic out of the cell. IV: modifying enzymes can inactivate the antibiotics by adding new functional groups. Figure adapted from Levy and Marshall, 2004.

Resistance to several drugs can arise from mutations affecting the drug target. Analysis of streptomycin resistant *Mycobacterium tuberculosis* has shown that the majority of these carry point-mutations in the *rpsL* gene, which encodes the S12 protein of the 30S ribosomal subunit, causing resistance to streptomycin (Sreevatsan *et al.* 1996).

Resistance to rifampicin has been shown to be caused by point-mutation in the *rpoB* gene, which encode the β subunit of the RNA polymerase, preventing binding of rifampicin to the polymerase. Mutations conferring resistance to rifampicin usually cause slower growth of the bacteria, but naturally slow-growing bacteria such as *Mycobacterium tuberculosis* does not seem to suffer from this (Mariam *et al.* 2004).

Target-site alterations have also been shown to confer resistance towards macrolides (Leclercq and Courvalin 1991), vancomycin (Cetinkaya *et al.* 2000), flouroquinolones (Hooper 2000). Often these target modifications only confer resistance towards a single class of compounds. However, a specific modification of the 23S RNA from the 50S subunit by a methyltransferase conferred resistance to all classes of antibiotics acting at this site (Long *et al.* 2006).

Antibiotic inactivation

The destruction of penicillin and cephalosporins by β-lactamases is the most wide-spread type of bacterial resistance. The β-lactamases cleave the β-lactam ring of penicillin and cephalosporin yielding the inactive derivates penicilloic acid and cephalosporanoic acid (Jacoby and Munoz-Price 2005).

Resistance to chloramphenicol is primarily due to the enzyme chloramphenicol acetyl transferase (CAT). The enzyme acetylates the primary hydroxyl group of the drug thereby inactivating it (Murray and Shaw 1997). Genes encoding CAT are widespread among both Gram-positives and Gram-negatives, and are often located on plasmids. Streptomycin and other aminoglycosides are often inactivated by phosphorylation, adenylation or N-acetylation (Wright *et al.* 1998).

Drug efflux pumps

Resistance towards tetracyclines are in most cases caused by tetracycline-specific efflux pumps, usually encoded by plasmid-borne genes. Several different tetracycline efflux pump genes have been identified. The efflux pumps are membrane-spanning multimer proteins; they actively pump tetracyclines out of the cytoplasm in exchange of proton (Ball *et al.* 1980). Other efflux pumps exist that target other antibacterial drugs, such as chloramphenicol and quinolones (Piddock 2006).

Resistance gene transmission

We saw in the previous section that some of the resistance mechanisms arise from mutations in chromosomal genes, such as resistance to streptomycin and rifampicin. This type of resistance can arise in pure cultures and resistant mutants can easily be selected by growing the bacteria in the presence of the drug. However, many types of resistance to clinically important drugs can clearly be transferred between bacteria of different genera. It is generally believed that many of the genes encoding drug-resistance arose from antibiotic-producing bacteria themselves. Antibiotic-producing bacteria such as the streptomycetes carry them in order to protect themselves from their own antibiotics (Benveniste and Davies 1973, Hopwood 2007). It is conceivable that these genes are being transferred among bacteria. Figure 4 illustrates some of the mechanisms by which this transfer can take place.

Conjugation

Multiple drug-resistance genes are often carried on so-called R-plasmids. Apart from the drug-resistance genes, these plasmids often carry genes that mediate conjugative transfer of the plasmid in a process similar to the F-plasmid. These transfer-genes encode specialized surface structures called ‘sex pili’, similar to those of the F-plasmid. A cell with sex pili (the donor) will attach itself to a cell without it (the recipient) and the cells will be pulled close together. A protein-pore will then form between the two cytoplasms and the R-plasmid will attach to the pore at a region called the *oriT* (origin of transfer).

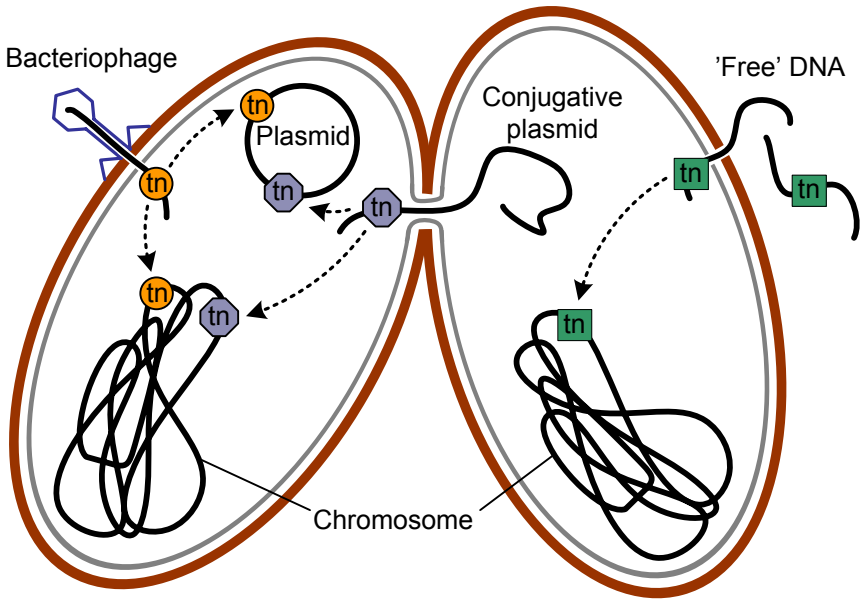

Figure 4. Drug resistance genes can be transferred between bacteria by a variety of mechanisms. By bacteriophages in a process called transduction; by conjugative plasmids, called conjugation and by 'naked' DNA, called transformation. Often the resistance genes are carried on transposons, easing the insertion into plasmid or chromosomal DNA of the recipient. Figure adapted from Levy and Marshall, 2004.

A single-stranded nick will be introduced at the *oriT* and the strand will be transferred in the 5' to 3' direction through the pore into the recipient. The single strands in both donor and recipient will be turned into double-stranded DNA by the DNA polymerase III (Franklin, Snow and Franklin 2005). It has been shown that conjugative transfer of resistance genes occurs with high frequency in the intestine of humans and animals (Shoemaker *et al.* 2001).

Transformation

Most bacteria can, under certain conditions, take up foreign DNA from the environment and incorporate it into their genome. The bacteria must be so-called competent; in this state they express proteins that will bind 'naked' double-stranded DNA and facilitate its uptake. This 'naked' DNA could stem from dead and lysed drug-resistant bacteria and thereby confer resistance to the recipient. Transformation appears to be the principal mechanism for transfer of resistance genes among streptococci and related genera (Hakenbeck 2000).

Transduction

Bacteriophages carrying resistance genes do not seem to be a normal source of resistance among bacteria. But they are likely to be involved in transfer DNA carrying resistance genes inside transposons or other mobile elements.

Transposons and integrons

Transduction and transformation requires, that the foreign DNA sequences are incorporated into the genome of the recipient; either into the chromosome or a plasmid. This could be accomplished by homologous recombination if the foreign DNA and the recipients DNA contain homologous sequences. However, since drug-resistance is transferred readily between different genera of bacteria, this is unlikely to be the only process. It has become clear that many drug-resistance genes are carried on DNA elements called transposons (Skurray and Firth 1997). Transposons can insert themselves into sequences with limited homology. The excision from one piece of DNA and insertion into another is performed by enzymes called transposases and is encoded by genes carried on the transposons themselves. The transposase enzyme recognizes insertion sequences (IS elements) at the ends of the transposon. These sequences appear in either direct or inverted order at each end. Transposons carry a variety of genes between these insertion sequences, often coding for drug-resistance. In some transposons these genes are arranged in structures called integrons. Integrons have conserved sequences on either side of the inserted genes; the sequences are recognized by integrase enzymes and allow for insertion of new genes into the integron (Franklin, Snow and Franklin 2005).

The above mentioned mechanisms accounts for the remarkable ability of bacteria to exchange genetic information such as drug-resistance genes. It has also been shown that sub-inhibitory doses of aminoglycoside and fluoroquinolone antibiotics induced transformation in *Streptococcus pneumonia* (Prudhomme *et al.* 2006) and that sub-inhibitory concentrations of penicillin increased the conjugal transfer of plasmid DNA from Escherichia coli to *Staphylococcus aureus* and *Listeria monocytogenes* in *in vitro* experiments (Trieu-Cuot *et al.* 1993). In light of the previously mentioned *modern view*

on antibiotics, it seems that the oxidative stress induced by sub-inhibitory concentrations of antibiotics results in competence for genetic transformation and activation of conjugal transfer systems, as part of the stress response.

In this light it seems that the current strategy of attempting to discover new drugs or to modify the old ones, in order to stay ahead of the bacteria, is bound to fail. New non-antibiotic approaches have therefore been suggested. I will describe two of these: anti-virulence drugs and treatments that involve stimulation or recruitment of the innate immune system of the host.

Antivirulence therapy

The difference between pathogenic and non-pathogenic strains of bacteria is usually determined by a small number of genes. The genes that enable a strain to infect and cause disease are known as virulence genes and the proteins they encode are known as virulence factors. Many of these virulence genes are located in pathogenecity islands (PAIs) on the chromosome or on plasmids. These virulence genes are often tightly regulated; the bacteria will sense their environment and under the right circumstances, i.e. inside the host, activate their virulence factors. Figure 5 gives an overview of the different virulence-targets that currently are being investigated.

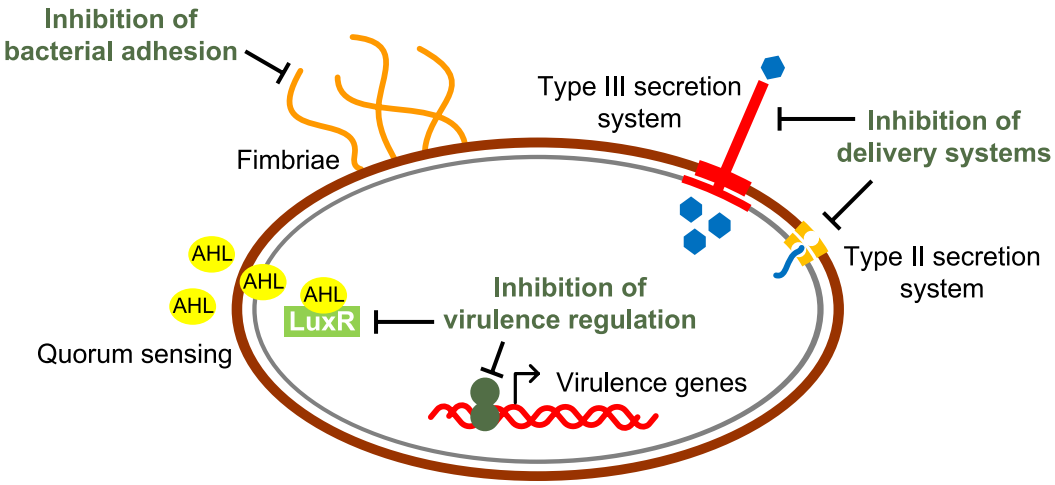

Figure 5. Some of the different virulence traits that can be targeted by antivirulence therapy. The figure was adapted from Clatworthy *et al*. 2007.

The idea behind antivirulence therapy is to target these virulence factors or their expression. This will prevent the pathogen from establishing an infection without affecting the growth of the bacteria which, as we have seen, inevitably would lead to resistance development (see figure 6).

One of the first steps in an infection is for the bacteria to adhere to host cells. Bacteria often use fimbriae or similar structures on their surface for this purpose (Hung *et al.* 1996). A class of compounds that target these fimbriae are called *pilicides* (Pinkner *et al.* 2006, Svensson *et al.* 2001). These compounds were found to target the fimbriae of pathogenic *Escherichia coli*, but could potentially be used for many Gram-negative bacteria. Pilicides inhibit the formation of the fimbriae by interfering with the chaperone-usher pathway necessary for their assembly (Sauer *et al.* 2000, Sauer *et al.* 2004).

Another target could be bacterial secretion systems. A wide range of bacteria use these systems for delivery of toxins or virulence effectors. *Vibrio cholerae* uses the type II secretion system (T2SS) for delivery of its toxin (Davis *et al.* 2000). The type III secretion system (T3SS) is a syringe-like structure that allows the bacteria to inject virulence effectors into eukaryote cells. *Escherichia coli* O157:H7 uses this system for delivery of Tir (translocated intimin receptor) into epithelial cells (LeBlanc 2003). A number of compounds have been identified that inhibit the T3SS of *Yersinia pseudotuberculosis* (Kauppi *et al.* 2003, Nordfelth *et al.* 2005). In many Gram-negative bacteria quorum sensing is used to regulate virulence genes. Quorum sensing is a mechanism whereby bacteria can sense their population density by releasing signal molecules. These can be acyl-homoserine lacton molecules (AHLs), which then interact with transcriptional regulators, such as LuxR, which then activate transcription of virulence genes (Miller and Bassler 2001). Analogs of AHLs have been found to inhibit virulence of *pseudomonas aeruginosa* (Muh *et al.* 2006, Smith *et al.* 2003).

Another example of inhibition of virulence gene regulation is the drug virstatin. It inhibits the expression of the toxin coregulated pili (TCP) and the cholera toxin (CT) in

V. cholerae by inhibiting dimerization of the transcriptional regulator ToxT (Hung *et al.* 2005, Shakhnovich *et al.* 2007). Oral administration of virstatin was shown to protect infant mice from colonization *V. cholerae*.

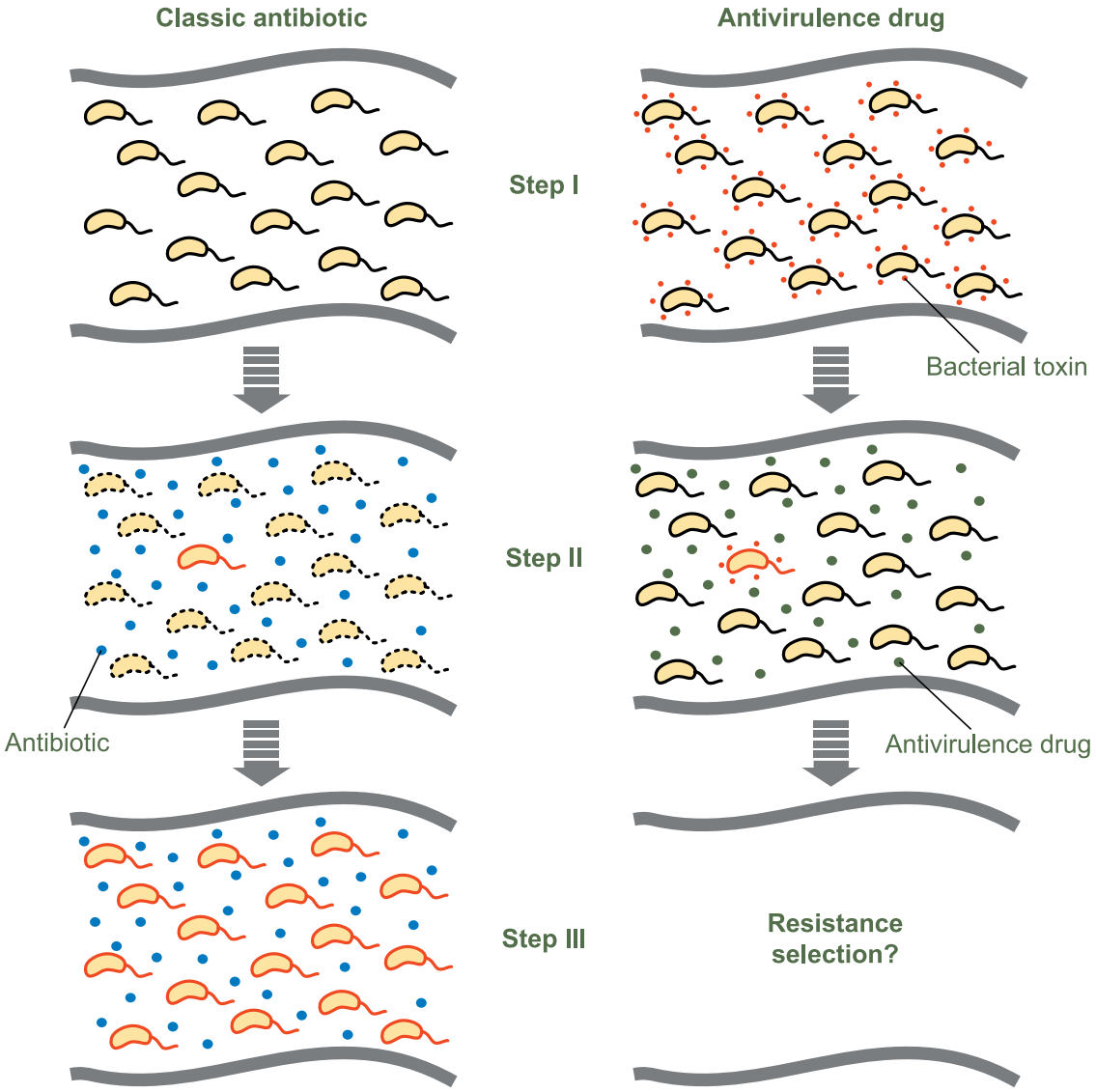

Figure 6. Classic antibiotics (left panel) selects strongly for resistance: in step one a pathogenic bacteria colonizes the host. Upon treatment with an antibiotic most of the bacteria dies, but a single bacteria is resistant. This could be from mutation or from acquired resistance. This lone resistant bacterium will now be able to divide and grow, because its non-resistant siblings/rivals have been killed by the antibiotic. In the right panel we again have pathogenic bacteria colonizing the host (expressing toxins). Upon treatment with an antivirulence drug the non-resistant bacteria cease production of toxins. We still will be faced with resistant bacteria, but because the drug does not kill the non-resistant siblings/rivals there is likely no selective advantage to being resistant.

Whether antivirulence therapy can live up to its promises still needs to be proven since none of the drugs have advanced beyond animal testing and pre-clinical trials.

Immuno-modulating drugs

When infected by a pathogen, humans will respond with a two-prone defence strategy: an immediate innate immune response and a slower, but more specific response from the adaptive immune response. The immediate response of the innate immune system involves activation of phagocytic and cytotoxic cells and recruitment of these to the site of the infection.

The innate immune response is triggered by receptors present in host cells. These receptors are called pattern recognition receptors (PRRs) (reviewed in (Medzhitov 2009)). They recognise evolutionary conserved molecular patterns common among all microorganisms, known as pathogen-associated molecular patterns (PAMPs). Well known examples of PAMPs are the lipopolysaccharide (LPS) of Gram-negative bacteria, the peptidoglycan of gram-positive bacteria and bacterial flagellin. The best known examples of PRRs are the transmembrane Toll-like receptors (TLRs) (Magalhaes *et al.* 2007). There are ten known TLRs in humans (TLR1-10) and they activate signalling pathways in the cells, when activated by PAMPs, resulting in induction of antimicrobial effectors and signal molecules that activate other parts of the innate immune system, as well as the adaptive immune response.

The PRRs do not distinguish between pathogenic or non-pathogenic bacteria. Despite the name, PAMPs are present in all microorganisms whether they are pathogenic or not. Instead the PRRs are present in compartments of the host that are not usually accessible to microorganisms. For example, TLR5, which detects bacterial flagellin, is present on the basolateral side of the epithelial cells in the gastrointestinal system. This space is normally not accessible to bacteria, only pathogens have the means (virulence factors) to gain access to this part of the host.

A number of drugs targeting these PRRs are in clinical and pre-clinical trials (reviewed in (Hancock *et al.* 2012)). Also, it has recently been reported that short synthetic peptides can act as modulators of the innate immune system (Nijnik *et al.* 2010, Scott *et al.* 2007). The peptides IDR-1 and IDR-1002 were shown to activate the immune response through a MAPK pathway. This resulted in activation and recruitment of monocytes and afforded protection in a mouse model against *Staphylococcus aureus*.

Caenorhabditis elegans

When searching for antivirulence, or immuno-modulating compounds, we cannot rely on *in vitro* models. *In vitro* models are not likely to replicate the condition inside a human host. Therefore, *in vivo* models, that replicate the human disease as closely as possible, are essential in order to investigate the effect of these drugs. During the past ten years *Caenorhabditis elegans* has emerged as a useful infection model for several pathogenic bacteria and fungi (reviewed in (Sifri *et al.* 2005)).

C. elegans is a small free-living nematode that lives in soil, where it feeds on microorganisms. There are two sexes, male and hermaphrodite. Hermaphrodites produce both eggs and sperm and are capable of self-fertilization as well as fertilization by a male. Males produce only sperm and must mate with a hermaphrodite in order to reproduce. When self-fertilizing, the hermaphrodite will produce approximately 300 progeny; when fertilized by a male, the male sperm will outcompete the hermaphrodite's own sperm and the ~1000 progeny produced will be the result of cross-fertilization (Hope 1999).

C. elegans life-cycle

Under laboratory conditions the growth of *C. elegans* is rapid. It will develop from fertilized egg to an adult capable of egg-laying in just three days at room-temperature. The hermaphrodite gonads first produce germ cells which differentiate as sperm (approximately 300 sperm cells are produced) before switching completely to egg production. The sperm cells are stored in the two spermathecas, where they will fertilize the passing eggs (see figure 7A).

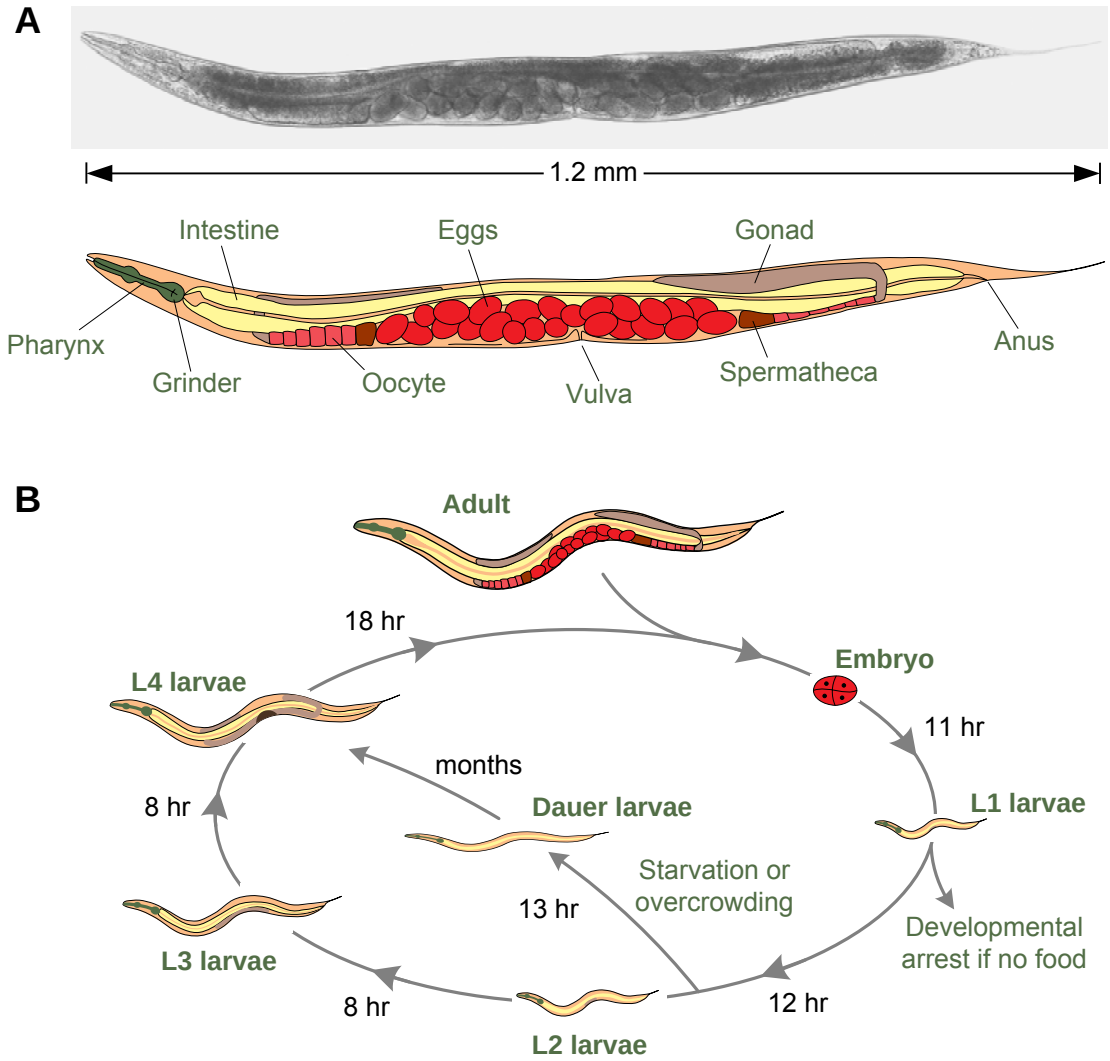

Figure 7. A: Anatomical features of *Caenorhabditis elegans*. The picture shows an adult hermaphrodite *C. elegans sek-1* (strain KU4) mutant. Drawing adapted from Alberts, 2008. **B:** Life-cycle of *C. elegans*. at 22°C. Drawing adapted from (Baylis and Vazquez-Manrique 2012).

The eggs hatch as larvae and these will develop into adults through four larval stages (L1 to L4), separated by moults. During each moult the larvae will synthesize a new outer cuticle under the old cuticle, and shed the old one. The worms live for a total of approximately three weeks, but they are only fertile for about four days.

Development from fertilization to hatching is referred to as embryogenesis; this takes about 14 hours (at room-temperature) and generates the first larval stage (L1) which is only 250 μm long, but has the same general structure as the adult worm (see figure 7B).

Over the next ~50 hours development will proceed through four moults and end up with an adult worm capable of reproduction. Under unfavourable environmental conditions (i.e. starvation) L2 larvae can moult into so-called *dauer* larvae. The cuticle of the dauer larva is completely sealed, protecting the larva from the environment. The dauer larva is able to live for three to four months without developing further. It will only moult into an L4 larva if a food source becomes available. The entry into this state is regulated by the insulin-like receptor pathway, which is also part of the innate immune system of the nematode (Schulenburg *et al.* 2004).

C. elegans immunity

As a soil-dwelling organism, grassing on microorganisms, *C. elegans* must have an innate immune system to protect it from infections. The first barrier is the tough outer cuticle layer that protects the worm from microbial penetration. A second barrier is the pharynx, which has two bulbs: the terminal bulb act as a grinder, which mechanically disrupts the ingested microorganisms so that very few intact microorganisms enter the intestinal tract. The few whole microorganisms that do enter are prevented from colonizing the worm by peristaltic movements, which serve to remove harmful microorganisms from the intestinal tract. *C. elegans* also possesses inducible immune pathways that resemble those of the innate immune system in mammals. These pathways are believed to control responses such as antimicrobial peptides, lysozymes and possibly other defence responses (Schulenburg, Kurz and Ewbank 2004, Sifri, Begun and Ausubel 2005).

There are a minimum of four pathways regulating immunity of *C. elegans*. These are the transforming growth factor-ß-like pathway, the p38 mitogen-activated protein kinase pathway (p38 MAPK pathway), the insulin-like receptor pathway, and the programmed cell death pathway (Schulenburg, Kurz and Ewbank 2004). Two of these are of particular interest, since they are known to be involved in the innate immune response of *C. elegans* towards bacterial infections of the intestine: the p38 MAPK pathway and the insulin-like receptor pathway (see figure 8). The p38 MAPK pathway has been shown to be induced in response to infection by specific human pathogens such as *P.*

aeruginosa (Troemel *et al.* 2006) and *Yersinia pestis* (Bolz *et al.* 2010). In contrast, the insulin-like receptor pathway is believed to provide the nematode with a continuous low-level protection against a broad range of pathogens (Shivers *et al.* 2008, Troemel, Chu, Reinke, Lee, Ausubel and Kim 2006). Apart from antimicrobial factors, these pathways also control the expression of proteins involved in protection against environmental stress.

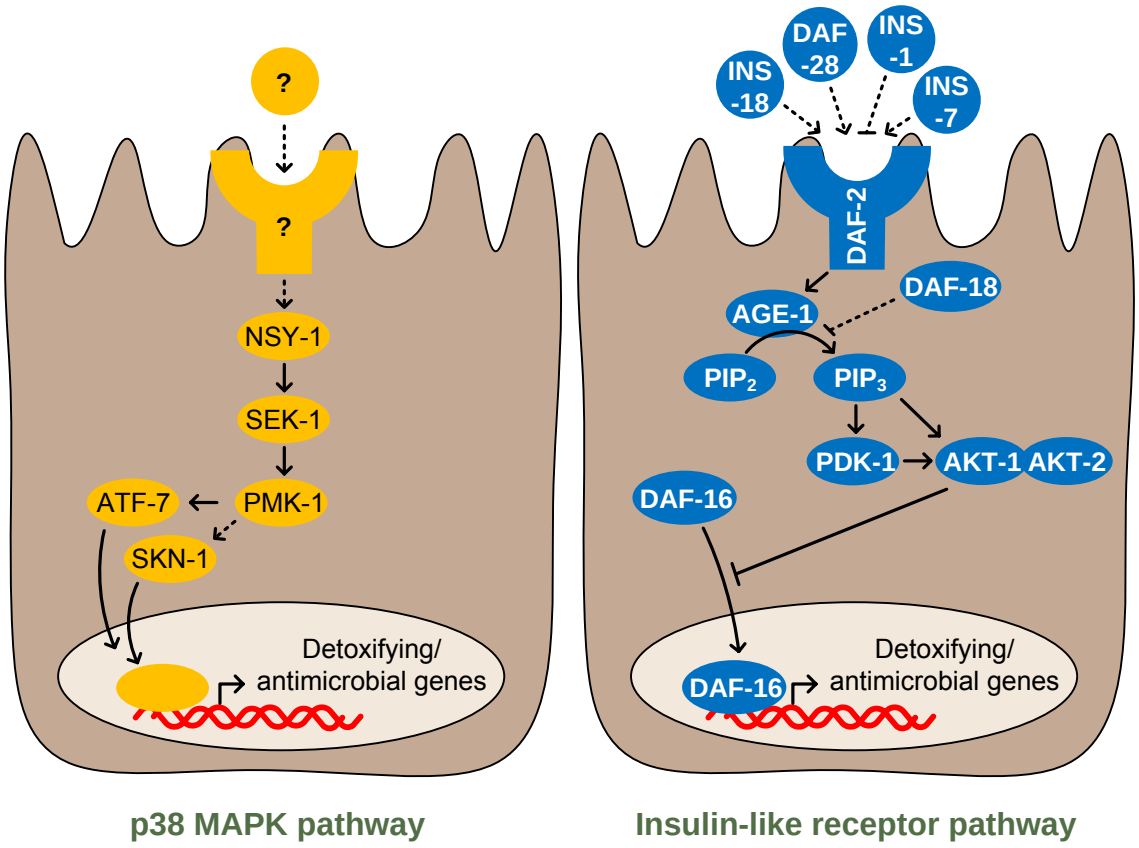

Figure 8. The signalling cascades involved in the p38 MAPK pathway and the insulin-like receptor pathway. Solid arrows indicate interactions that have been verified to be implicated in immunity. Dotted arrows indicate relationships that are still uncertain. Figure adapted from (Schulenburg, Kurz and Ewbank 2004).

It is not known exactly how these pathways are activated by pathogens. No pattern recognition receptors (PRRs) have yet been identified in *C. elegans*. However, it was recently reported that the immune response of *C. elegans* towards *staphylococcus aureus* was not dependant on the use of live bacteria (Irazoqui *et al.* 2010). This

indicates that pathogen-associated molecular patterns (PAMPs) could be involved in activation of the innate immune system of the nematode.

C. elegans as an infection model

Because the worm is a natural bacterivore, there is no need for injecting or otherwise force bacteria into it. One simply replaces the worm's normal laboratory food source of non-pathogenic *E. coli* OP50 with the pathogen of choice. Usually as a lawn of bacteria on an agar plate that the worm are allowed to crawl around and feed on. A number of human pathogens have been shown to strongly reduce the lifespan of the nematode (Sifri, Begun and Ausubel 2005). This has been interpreted as a result of a pathogenic process similar to that in humans. It has been suggested that virulence of bacteria developed, in fact, as a defence against microbivores such as *C. elegans* (Vaitkevicius *et al.* 2006). When feeding on *E. coli* OP50 *C. elegans* has a life-span of about two weeks; this is significantly shortened when grown on these pathogenic bacteria. The killing process can be divided into *slow killing* where live bacteria (which have passed intact through the pharynx) accumulates in the intestine of the worm and over time kills it, and *fast killing* where the killing is mediated by toxins secreted by the bacteria. This simple feeding-based pathogenesity model has made *C. elegans* an attractive model for screening of antimicrobials (Breger *et al.* 2007, Moy *et al.* 2006), but also for identification of immuno-modulating drugs (Pukkila-Worley *et al.* 2012).

Project 1 (unpublished data)

Peptide inhibitors of *Vibrio cholerae* virulence.

The Gram-negative bacteria *Vibrio cholerae* is the causative agent of the diarrhoeal disease cholera (Sack *et al.* 2004). The bacteria lives in coastal waters and has been endemic to southern Asia for more than 1000 years, but has also caused seven pandemics over the last 200 years.

The two best studied virulence factors of *V. cholerae* are the toxin co-regulated pili (TCP) and the cholera toxin (CT). The expression of both of these is controlled by the transcriptional regulator ToxT (Prouty *et al.* 2005). A drug that could inhibit the function of ToxT, would therefore be a useful antivirulence drug against *Vibrio cholerae.* Virstatin is an example of such a drug; it inhibits the expression of TCP and CT in *V. cholerae* by inhibiting the dimerization of ToxT. (Hung, Shakhnovich, Pierson and Mekalanos 2005, Shakhnovich, Hung, Pierson, Lee and Mekalanos 2007). See also figure 9.

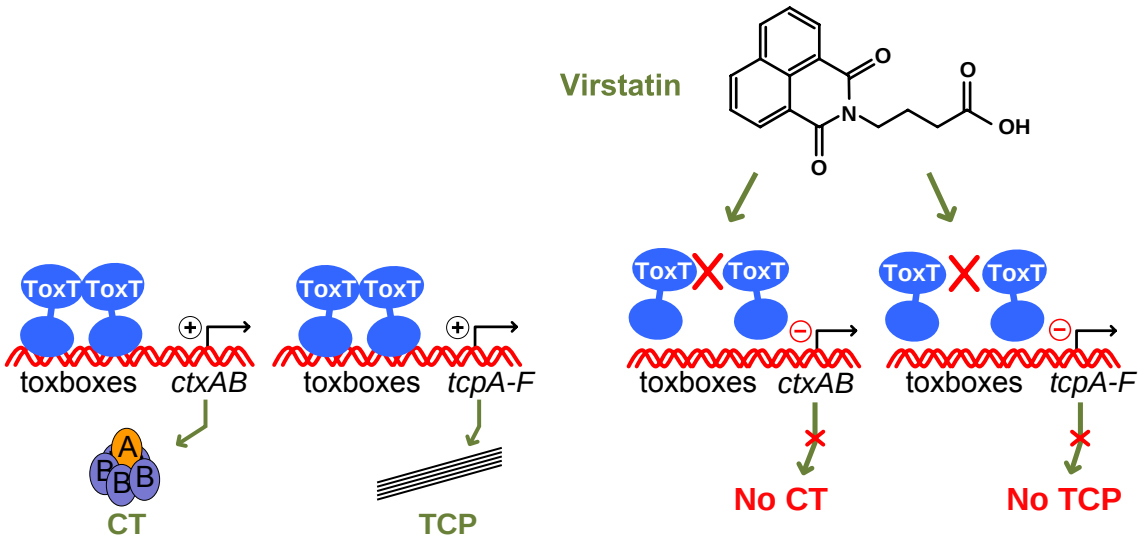

Figure 9. The small bioactive molecule virstatin (4-[N-(1,8-napthalimide)]-n-butyric acid) prevents ToxT from forming the active dimer, thereby inhibiting transcription of both the cholera toxin (CT) and the toxin co-regulated pili (TCP).

Hung *et al*. constructed a *V. cholerae* strain that carried a chromosomally located tetracycline-resistance gene under control of the cholera toxin promoter. This strain was screened for tetracycline sensitivity (i.e. inhibition of the cholera toxin promoter) when grown in the presence of different small-molecule drugs. This construction was used to screen a commercially available library of 50,000 small molecules. Virstatin was shown to inhibit the production of CT and TCP, without affecting the growth rate of wild-type *V. cholerae* strains. They also showed that virstatin inhibited the ability of *V. cholerae* to colonize the small intestine of infant mice. They tested the ability of a wild-type *V. cholerae* to colonize the small intestine, with and without virstatin. In the presence of virstatin, the number of *V. cholerae* that was recovered from the mice intestine was only 0.01% of that recovered from the intestine of untreated mice.

Inspired by this study, we decided to try to find other inhibitors of *Vibrio cholerae* by targeting ToxT. We decided to screen a plasmid library encoding approximately 500.000 small circular peptides (9 amino acids long with 6 randomized amino acids). Such peptides could conceivably act in a manner similar to that of virstatin, and interfere with the expression of virulence factors.

This library was constructed by Susanne Kjelstrup from University of Copenhagen and utilises the SICLOPPS (split intein-mediated circular ligation of peptides and proteins) system which can be used to generate libraries of cyclic peptides. An intein is a segment of a protein that is able to splice itself out and rejoin the flanking segments (called exteins) with a peptide bond. These types of *cis*-splicing intein-containing proteins are transcribed from a single gene. In the case of split inteins, two genes are involved. The SICLOPPS system uses the DnaE split intein from the cyanobacterium *Synechocystis* sp PCC6803. DnaE is the catalytic subunit alpha of DNA polymerase III and it is encoded by two separate genes, *dnaE-n* and *dnaE-c*. The *dnaE-n* product consists of a 774 amino acid (aa) N-extein region followed by a 123-aa intein region (I_N), and the *dnaE-c* product consists of a 36-aa intein region (I_C) followed by a 423-aa C-extein region (Wu *et al.* 1998). During *trans*-splicing the two extein regions are joined together to form the complete DnaE protein. In SICLOPPS the two intein regions are rearranged to form a

cis-intein: I_C:target peptide:I_N (Evans *et al.* 2000, Scott *et al.* 1999). This will result in the target peptide being released from the inteins and cyclized (see figure 10).

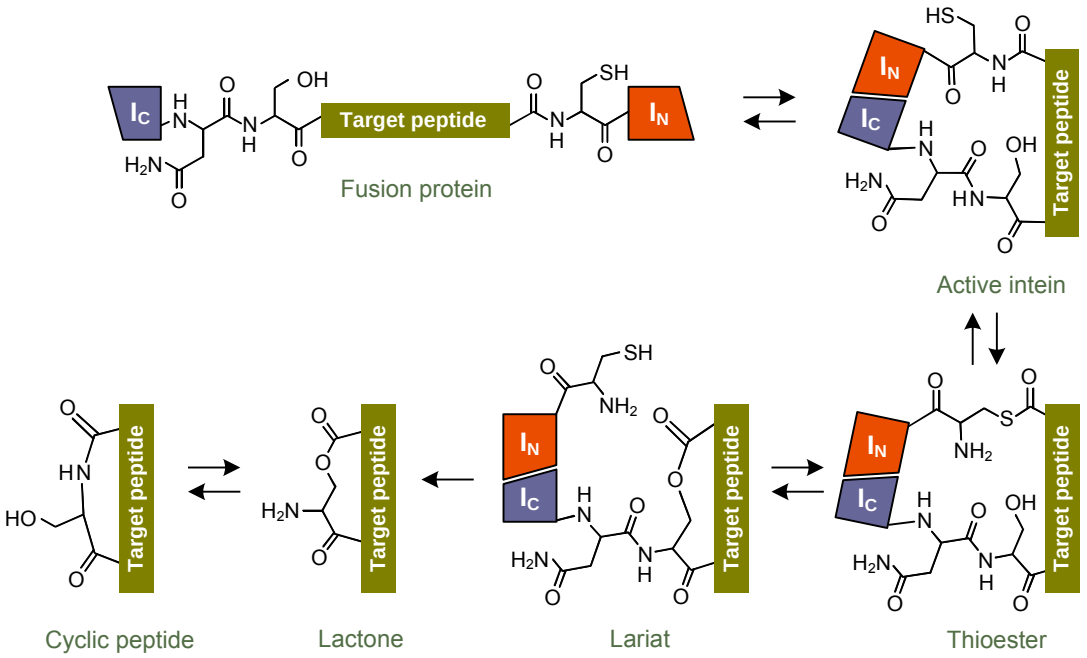

Figure 10. The SICLOPPS mechanism. The two intein regions folds to form the active intein. The cystein at the IN:target peptide junction undergoes an N-to-S acyl shift to form a thioester. The thioester undergoes transesterfication, by the serine at the IC:target peptide junction, to form the lariat intermediate. The asparagine side-chain cyclization liberates the cyclic peptide as a lactone. An O-to-N acyl shift generates the thermodynamically favoured lactam product. Figure adapted from Tavassoli and Benkovic, 2007.

A wide range of cyclic peptides or proteins can be expressed by the SICLOPPS system. Scott *et al.* showed that the *E. coli* enzyme dihydrofolate reductase (DHFR) could be expressed, and the cyclic DHFR was shown to be more resistant to proteolysis than the native linear protein (Scott, Abel-Santos, Wall, Wahnon and Benkovic 1999). It is conceivable that small cyclic peptides would also be more resistant to cellular catabolism than similar linear peptides. Since the splicing is only dependant upon the chemistry of the inteins and does not require the aid of proteases, the system can be carried on a plasmid vector and be utilized in a variety of hosts.

The plasmid library was constructed by inserting degenerate oligonucleotides in between the I_C and I_N genes, on a SICLOPPS vector, thereby creating an open reading

frame encoding the desired fusion protein, under control of a IPTG inducible *lac* promoter derivate ($P_{A1/O4}$). The target peptides will contain six random amino acids plus the three amino acids needed for the cyclization. The plasmid library was given to us as a mixture (i.e. a miniprep) of plasmids encoding approximately 500.000 different combinations of peptides.

Because each plasmids needs to be transformed into a cell, for its peptide to be expressed, we needed to design *reverse screening system*. In such a screen a cell expressing a functional peptide (capable of inhibiting ToxT-dimerization) would survive, whereas cells containing plasmids expressing non-functional peptides would die. Figure 11 gives an overview of our reverse screening system and shows how it works with our positive control – virstatin.

Materials and methods

Vector pToxT

We amplified the toxT gene from *Vibrio cholerae* Bah-2 with primers: 5'-GATACACCC-GGGAACTTTACGTGGATGGCTC-3' and 5'-GATACACCCGGGGGAACGATTTGAATCGATGTC-3'. The fragment was digested with *Xma*I and inserted into the vector pBAD18. The AraC-toxT fragment was amplified from pBAD18-toxT with primers: 5'-GATACAGACGTCTGA-CAGCTTATCATCGATGC-3' and 5'-GATACAGACGTCAGGGTTATTGTCTCATGAG-3' and inserted into the low copy vector pMW119 with AatII to create pToxT

Vector pMnt-P_{tcpA}

The P_{ant}/O_{mnt} promoter/operator was amplified from pPY97 (Lucchesi *et al.* 1986) with primers: 5' -TGCTAACCAGTAAGGCAAC-3' and 5'-GATACAATCGATGTGTATTGACATGATAG-AAGC-3' and inserted into pACYC184 with *Ava*I and *Cla*I. Then *mnt* was amplified from pPY97 with primers: 5'-GATACACCCGAGGGTACCGACGTCGGGCCCCATATGGAGCT-CACACTAACTTGGAGTGATGG-3' and 5'-GATACACTCGGGCGGTTGCCGCCGGGCGTTTTTTA AAATTAACCCGCCGTCAG-3' and inserted into pACYC184-P_{ant}/O_{mnt} with *Ava*I. The P_{tcpA} promoter (with toxboxes) was amplified from *Vibrio cholerae* Bah-2 with primers: 5'-G-

ATACAGGTACCCACTCCGGAAATATTTTAACGC -3' and 5'-GATACACATATGGGAAATGCATTG-CTTGGTCC-3' and inserted into pACYC184-P_{ant}/O_{mnt}-Mnt with *Kpn*I and *Nde*I to create vector pMnt-P_{tcpA}.

Both vectors were eletroporated into *E. coli* MC1000. Figure 11 gives a overview of these constructs.

Virstatin assay

An overnight culture of *E. coli* MC1000/pToxT+pMnt-P_{tcpA} was split into three 5 ml tubes. One with 8 µg/ml tetracycline, one with 8 µg/ml tetracycline+0.2% arabinose and one with 8 µg/ml tetracycline+0.2% arabinose+1000 µM virstatin. After 5 hours shaking at 37°C the cultures were diluted to 10.000x and 100 µl plated on plates with the same components as the liquid cultures.

Peptide screen

Eletrocompetent *E. coli* MC1000/pToxT+pMnt-P_{tcpA} were electroporated with '9aa peptide library miniprep DNA'. After eletroporation the cells are allowed to express the peptide in LB+1 mM IPTG for 1 hour at 37°C before we added LB+8 µg/ml tetracycline+1 mM IPTG+ 0.2% arabinose (plus relevant antibiotics for the three plasmids) and incubated at 37°C over night (ON).

10 µl ON culture was plated on LB-agar+8 µg/ml tetracycline+1 mM IPTG+ 0.2% arabinose and incubated ON at 37°C. The next day the colonies on the plate were collected and plasmid was isolated with Qiagen miniprep procedure. The resulting plasmid prep was digested with *Asi*SI and *Pac*I (in order to linearize pToxT and pMnt-P_{tcpA}. This prep was then eletroporated into fresh *E. coli* MC1000/pToxT+pMnt-P_{tcpA} and the selection repeated in order to enrich the prep with plasmids encoding ToxT inhibitor peptides.

After 4 rounds of enrichment the fifth eletroporation was split in two: one receiving IPTG and one without IPTG and the plated on plates with and without IPTG in order to assess the effect of the enrichment.

Results and discussion

We tested our reverse screen using virstatin as a positive control. The screening strain *E. coli* MC1000/pToxT+pMnt-P_{tcpA} was able to grow on 8 μg/ml tetracycline without arabinose (no ToxT expression from P_{BAD}). In the presence of 0.2% arabinose, the strain was not able to grow on 8 μg/ml tetracycline due to ToxT activating expression of the Mnt-repressor, which in turn prevents expression of the tetracycline resistance gene *tetA*. In the presence of 1000 μM of virstatin the screening strain was returned to growth levels without arabinose-induction of ToxT. This indicated that ToxT was successfully inhibited by virstatin.

There were, however, two major problems with the assay: there was a clear background on the plate with tetracycline and arabinose. We attribute this to the presence of 'arabinose-resistant' cells, i.e. cells that lack arabinose-transporters and therefore do not respond to the inducer. Siegele and Hu have described this phenomenon of mixed populations, showing different levels of induction of the P_{BAD} promoter, sometimes dubbed 'all-or-nothing induction' (Siegele and Hu 1997). The other problem was the high concentration of virstatin needed to successfully inhibit ToxT. We attempted to lower the concentration of arabinose, but this only increased the background growth to unacceptable levels. We then attempted to insert the *lacY*A177C gene onto the pToxT plasmid. This gene encodes a mutated LacY transporter that allows arabinose into the cell (Bowers *et al.* 2004). This solution solved the background-problem with arabinose-resistant cells; however, we were no longer able to rescue the bacteria with virstain, even with lower concentrations of arabinose (data not shown).

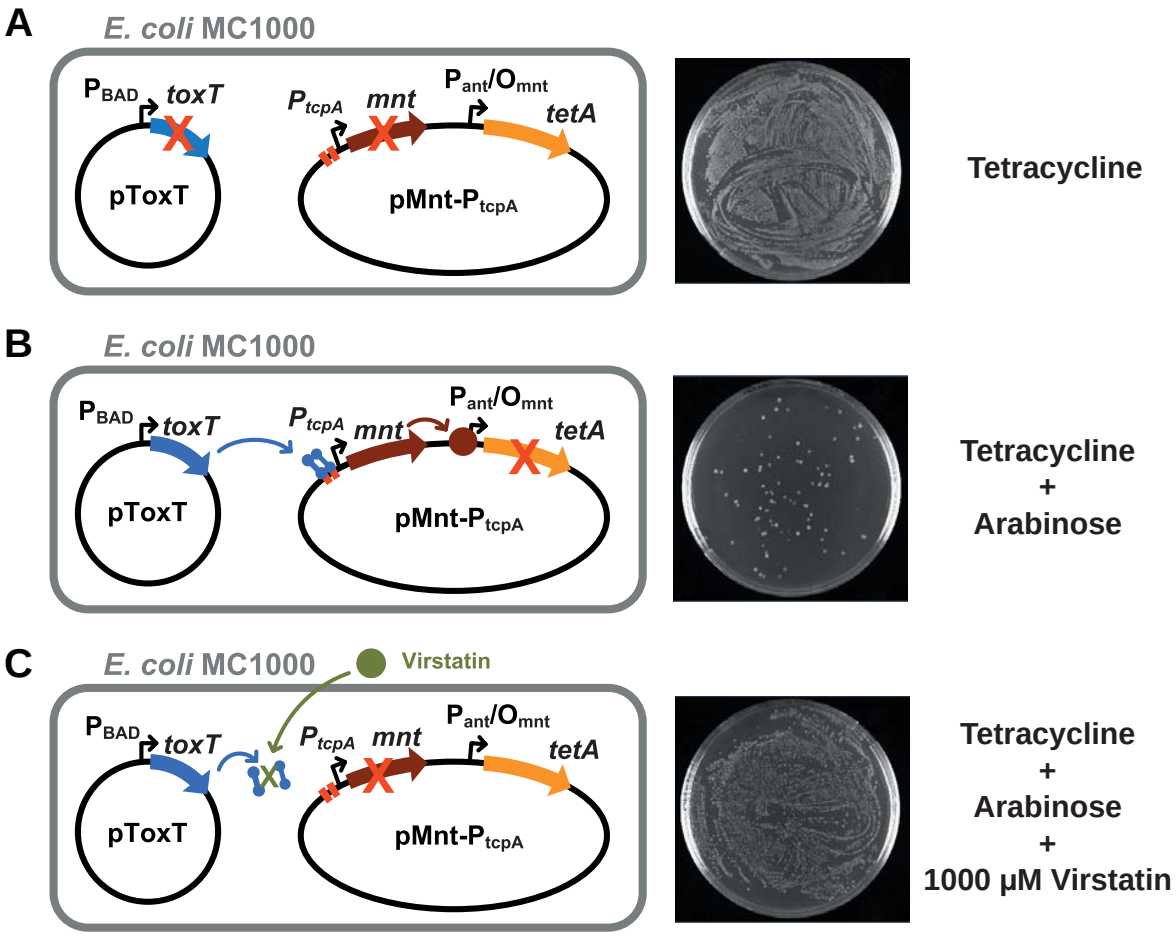

Figure 11. A: In the absence of the inducer arabinose, ToxT is not expressed from the P_{BAD} promoter. Without ToxT there is no expression of Mnt from the P_{tcpA} promoter, and TetA is expressed from the P_{ant} promoter – and the cells are tetracycline resistant. **B:** With arabinose in the plate ToxT is expressed from the P_{BAD} promoter. ToxT dimerizes and binds to the toxboxes, recruits the RNA polymerase to P_{tcpA} and we get expression of Mnt. Mnt binds to its operator O_{mnt} and the P_{ant} promoter is blocked which results in tetracycline sensitivity. **C:** ToxT is not able to dimerize and bind to the toxboxes because of virstatin. This prevents expression of Mnt and the P_{ant} promoter is open which results in tetracycline resistance.

We decided to use the original screening strain to screen the peptide library. We reasoned that maybe the peptides that expressed intracellular and in high numbers would be enough to inhibit the high number of ToxT molecules. Figure 12 shows the result. After 4 rounds of enrichment, we plated the strain onto agar-plates with or without IPTG. This would reveal if expression of the peptides made any difference to the survival rates. It turned out that there was only a very small difference. We

concluded that none of the selected peptides were able to inhibit ToxT. The colonies on the plates were likely 'background'.

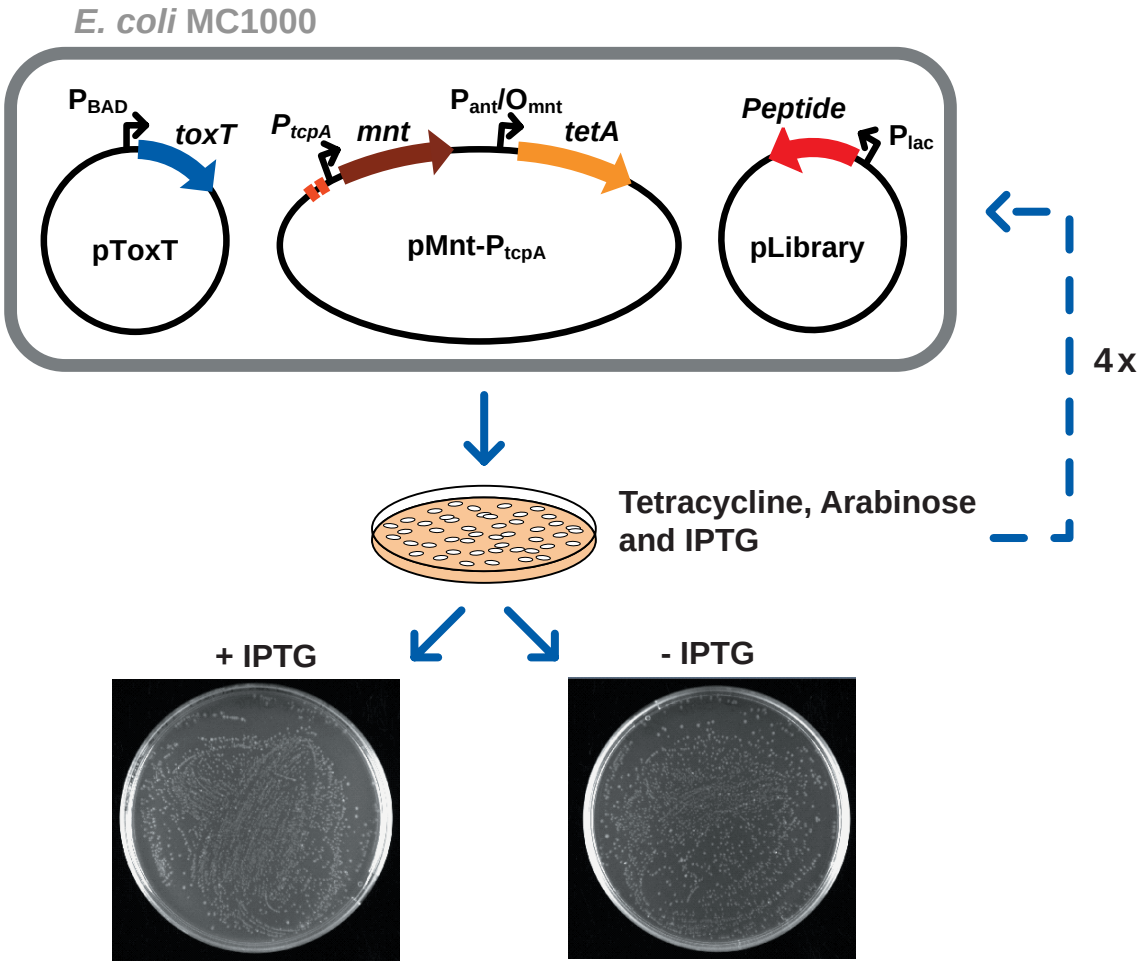

Figure 12. The peptide library was electroporated into *E. coli* MC1000/pToxT+pMnt-P_{tcpA}. After selection on plates the colonies were washed off and plasmid purified. pToxT and pMnt-P_{tcpA} linearized with restriction enzymes *Asis*I and *Pac*I. pLibrary was then reintroduced into fresh cells for a new round of selection. After four rounds of selection, the pLibrary plasmids were recovered and retransformed once again, but this time selected on plates with and without IPTG. We expected more cells on the plate with IPTG. However, this was not the case.

We conclude that the assay was unsuccessful. We ascribe this to two obvious reasons: 1. that there were no peptides in the library capable of inhibiting ToxT. 2. That the screen was not sensitive enough to select the peptides that worked. The control experiment with virstatin revealed that our assay was not as sensitive as we had wished. We needed to increase the concentration of virstatin to 1000 µM in order to rescue the

bacteria. We speculate that perhaps ToxT did not have the proper conditions for dimerization and activation of transcription from the P*tcpA* promoter. The screen was carried out in *E. coli* rather than in *V. cholerae* because *V. cholerae* are notoriously difficult to transform with high frequency. It would not have been possible to perform the assay in *V. cholerae*, but perhaps we could have improved the conditions for ToxT in *E. coli* by adding bicarbonate (Abuaita and Withey 2009) or bile salts (Hung and Mekalanos 2005) to the medium. It has been shown that ToxT, like many other members of the AraC family of activators, contains a ligand-binding domain for response to environmental factors (Prouty, Osorio and Klose 2005). It is possible that such environmental factors are needed for proper function of ToxT, and in the abscence of these factors too many molecules of ToxT are needed to activate transcription from the P_{tcpA} promoter. This in turn results in expression of too many ToxT molecules for virstatin and the peptides to handle.

Project 2 (see attached manuscript 1)

Lack of the RNA chaperone Hfq attenuates pathogenicity of several *E. coli* pathotypes towards *C. elegans*

The prokaryotic RNA-binding protein Hfq plays a vital role in posttranscriptional control in many bacteria. Hfq acts as a chaperone by pairing mRNAs and small RNAs (sRNA). This pairing between an mRNA and its corresponding sRNA can result in a variety of outcomes. Translation of the mRNA can be repressed or activated or the mRNA can be irreversibly inactivated by degradation (see figure 13) (De Lay *et al.* 2013, Vogel and Luisi 2011).

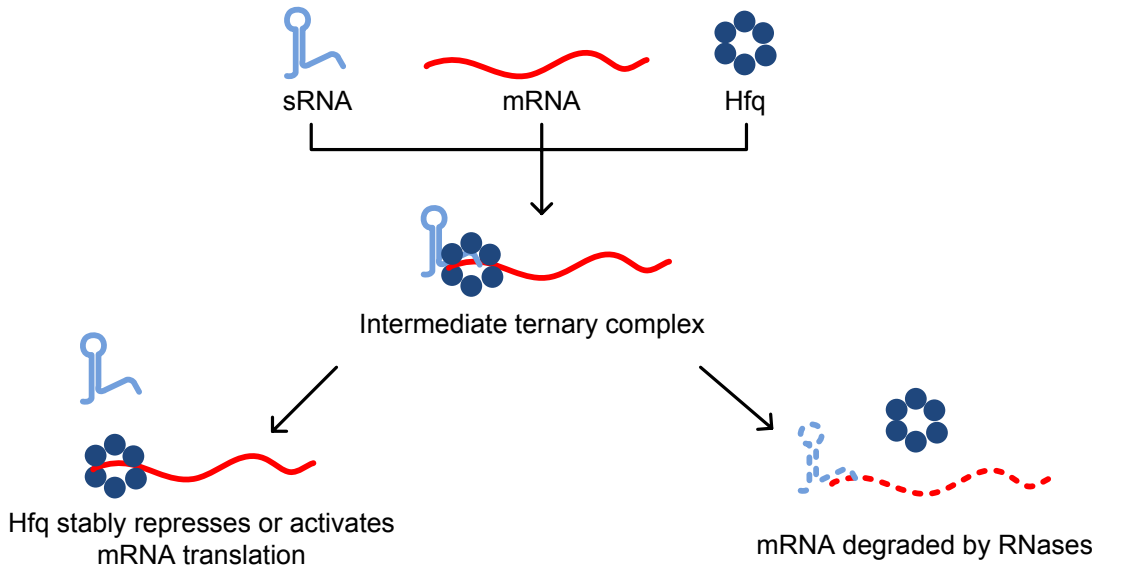

Figure 13. The different fates of mRNA after hfq-mediated binding to its small RNA (sRNA). Figure adapted from Vogel and Luisi, 2011.

Hfq has been shown to regulate virulence genes as well as core genome genes (Chao and Vogel 2010). It is interesting that many *hfq* mutants of pathogenic bacteria are strongly impaired in *in vivo* models of infection. They are often less capable in handling the stress associated with many host environments (Chao and Vogel 2010). This involvement of Hfq in virulence, paired with the recent finding that Hfq was vital for

pathogenesity of adherent invasive *E. coli* (AIEC), prompted us to look into the effect of Hfq on more types of pathogenic *E. coli*.

Results and discussion

We constructed *hfq* mutants of prototypic strains of verotoxin producing *E.* coli (VTEC, EDL933), enteroagregative *E. coli* (EAEC, 17-2) and uropathogenic *E.* coli (UPEC, J96). These strains were tested in the *C. elegans* infection model (see figure 14).

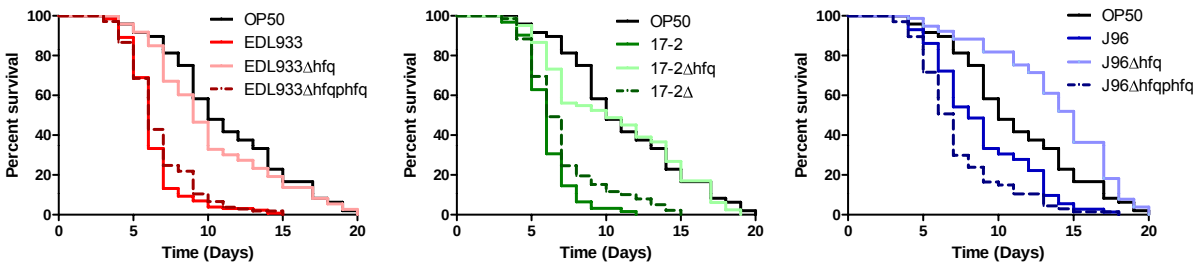

Figure 14. Lack of Hfq in all three *E. coli* pathotypes resulted in significantly increased lifespans of *C. elegans*. The increase in LT50 (3, 4 and 7 days) for VTEC, EAEC and UPEC strains was fully complemented in trans.

We can see that the UPEC strain J96 seems more affected by the lack of Hfq. This difference was also seen between the strain's ability to cope with oxidative stress, in the form of hydrogen peroxide. This could be due to slight variations in the core genome of the chosen strains. However, it is tempting to suggest that these different strains have experienced stepwise additions of virulence factors, as they evolved to survive in different niches, and that this acquired genetic material is to some extent regulated by Hfq in concert with sRNAs. Targeting Hfq has been proposed as a new approach against pathogenic bacteria (Wilson *et al.* 2007).

Project 3 (unpublished data)

Small scale high-throughput screen for inhibitors of Hfq

Inspired by the results in project 2, we decided to conduct a high-throughput screen for inhibitors of Hfq. We reasoned that since Hfq functions as a hexamer (De Lay, Schu and Gottesman 2013) breaking the protein-protein interactions between the individual Hfq monomers would inhibit its function. Hfq was tested in a two-hybrid screen (Karimova *et al.* 1998). In this screen the interaction between two hfq subunits produces an active adenylate cyclase which activates transcription of the *lac* operon (see figure 15).

Materials and methods

Vectors pUT18C-hfq and pKT25-hfq

The *hfq* gene was amplified from *E. coli* MG1655 with primers 5'-GATACAGGATCCCA-TGGCTAAGGGGCAATC-3' and 5'-GATACAGAATTCTTATTCGGTTTCTTCGCTG-3' and cloned into vectors pUT18C and pKT25 (BATCH system kit, Euromedex) with restriction enzymes *Bam*HI and *Eco*RI. The resulting vectors were electroporated into *E. coli* HB101. The vectors were tested by streaking the strain on plates with 1 mM IPTG and 40 μg/ml of X-gal. *E. coli* HB101 with the empty vectors and with vectors pUT18C-zip and pKT25-zip (containing a leucine zipper motif) were used as negative and positive controls.

Compound screen

Using the *E. coli* BTH101/pUT18C-*hfq*+pKT25-*hfq* strain, we performed a screen of two compound libraries: the 1280 pharmacologically active compounds $LOPAC_{1280}$ library and the MicroSource: FDA-approved US drug collection containing 1040 compounds. The screen was performed by the University of Massachusetts Medical School Small Molecule Screening Core Facility which also performed the plate reading and data analysis. The two-hybrid screen is based on the method devised by Karimova

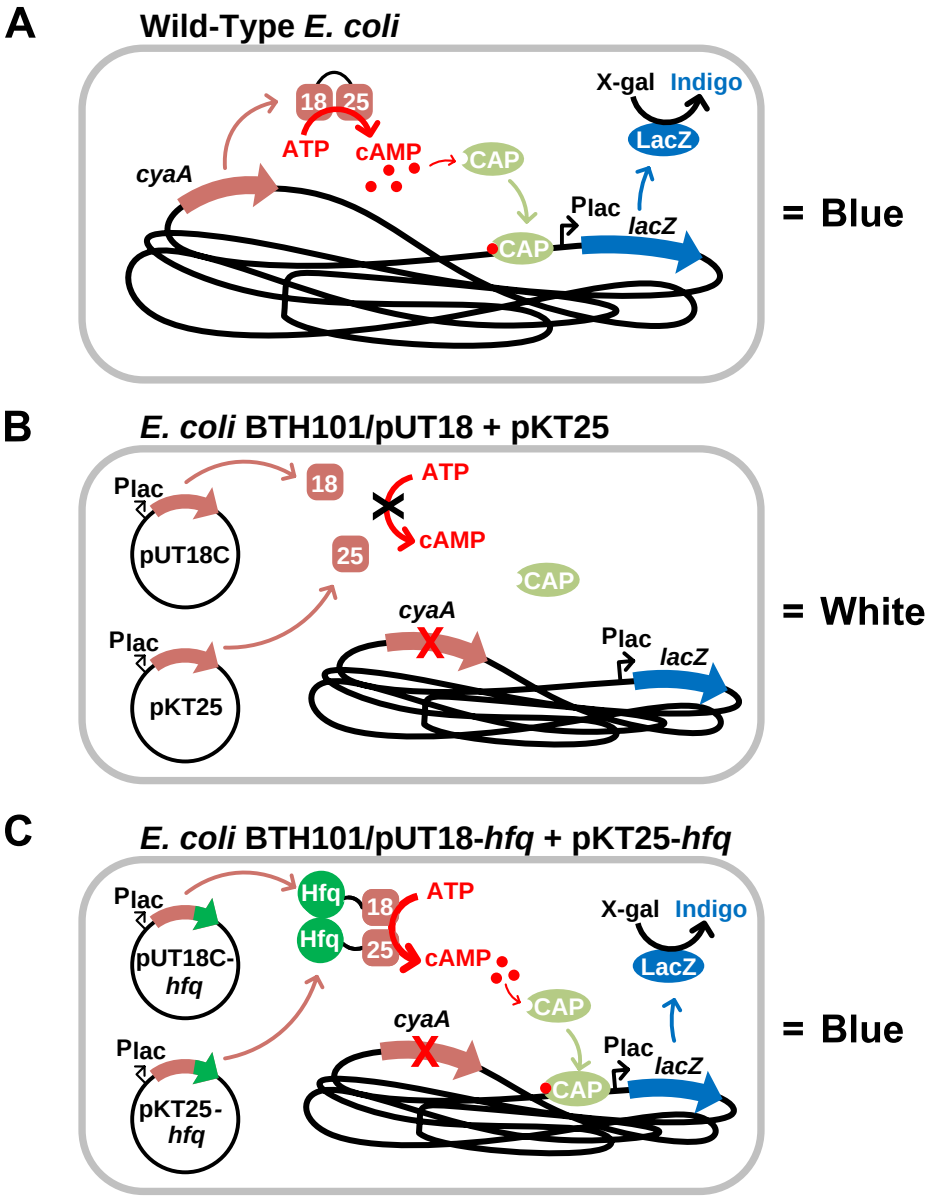

Figure 15. The figure shows the principle behind the 2-hybrid screen. **A:** in a wild-type *E. coli* the adenylate cyclase is an enzyme comprised of two subunits (T18 and T25). In the absence of glucose, the adenylate cyclase converts ATP into cAMP. cAMP activates the catabolite activator protein (CAP) which binds to the CAP-site in front of the *lac* operon and other operons for alternative energy-sources, activating the operons. In the presence of IPTG, LacZ will be expressed and converts X-gal (5-bromo-4-chloro-3-indolyl-β-D- galactopyranoside) into the blue 4-chloro-3-brom-indigo. **B:** In the *E. coli* BTH101 strain the *cya* gene has been deleted. The two subunits of the adenylate cyclase are expressed by the plasmids pUT18C and pKT25, but without a stable interaction between the two subunits cAMP will not be produced and the *lac* operon will remain inactive. This results in white (colourless) colonies on LB medium and no growth on maltose (the *mal* operon is also activated by CAP). **C:** If a pair strongly interacting proteins, like Hfq, are fused to the c-terminal of the T18 and T25 subunits, they are brought together and form a functioning adenylate cyclase. This will result in blue colonies on X-gal.

et al (Karimova, Pidoux, Ullmann and Ladant 1998) where a stable interaction between the fusion subunits produces an active adenylate cyclase which activates transcription of the *lac* operon (see figure 15).

Overnight cultures of *E. coli* BTH101/pUT18C-*hfq*+pKT25-*hfq* was diluted to about 10^3 cells/200 µl and arrayed in 96 well plates in 200 µl of Davis and Mingioli minimal medium (Davis and Mingioli 1950) supplemented with 0.2% maltose, ampicillin (50 ug/ml), kanamycin (25 mg/ml), thiamine (2 µg/ml), X-Gal (40 µg/ml) and IPTG (0.5 mM). The plates were incubated at 30°C and scanned daily at a wavelength of 664 nm on a Tecan Safire plate reader.

Results and discussion

We inserted *hfq* into pUT18C and pKT25 and verified the interaction by streaking the cells on an agar-plate with X-gal. We used a negative control-strain with empty pUT18C and pKT25 vectors and a pre-constructed strain with a leucine-zipper construct (pUT18C-zip and pKT25-zip) as the positive control (see figure 16).

We screened two smaller compound libraries (the 1280 pharmacologically active compounds $LOPAC_{1280}$ library and the MicroSource: FDA-approved US drug collection, comprised of 1040 compounds). We identified one possible candidate, nordihydroguaiaretic acid (NDGA). As a verification of our hit, we conducted a survival assay in the *C. elegans* infection model. We choose the UPEC strain J96, as this had shown the strongest dependency on Hfq, in project 2. We expected to see a lifespan extension similar to the 7 days seen between the wild-type and the *hfq* mutant. However, the result was far less than expected (see figure 17).

It is difficult to figure out why NDGA apparently do not inhibit the function of Hfq *in vivo*, even at a very high concentration. Perhaps NDGA is not able to enter into the nematode and exert its effect on the bacteria inside the worm. It could also be that the CyaA-Hfq fusion proteins used for the screen do not resemble the natural Hfq. When constructing the fusion we trusted that only a properly folded Hfq fused to CyaA would

interact and produce blue colonies on X-gal. However, it is possible that CyaA distorts the conformation of the N-terminal part of Hfq. It has been reported that the N-terminus of Hfq is responsible for the function of Hfq, whereas the C-terminal is largely redundant (Olsen *et al.* 2010, Vecerek *et al.* 2008). So, perhaps we have selected inhibitors against a non-functional conformation of Hfq.

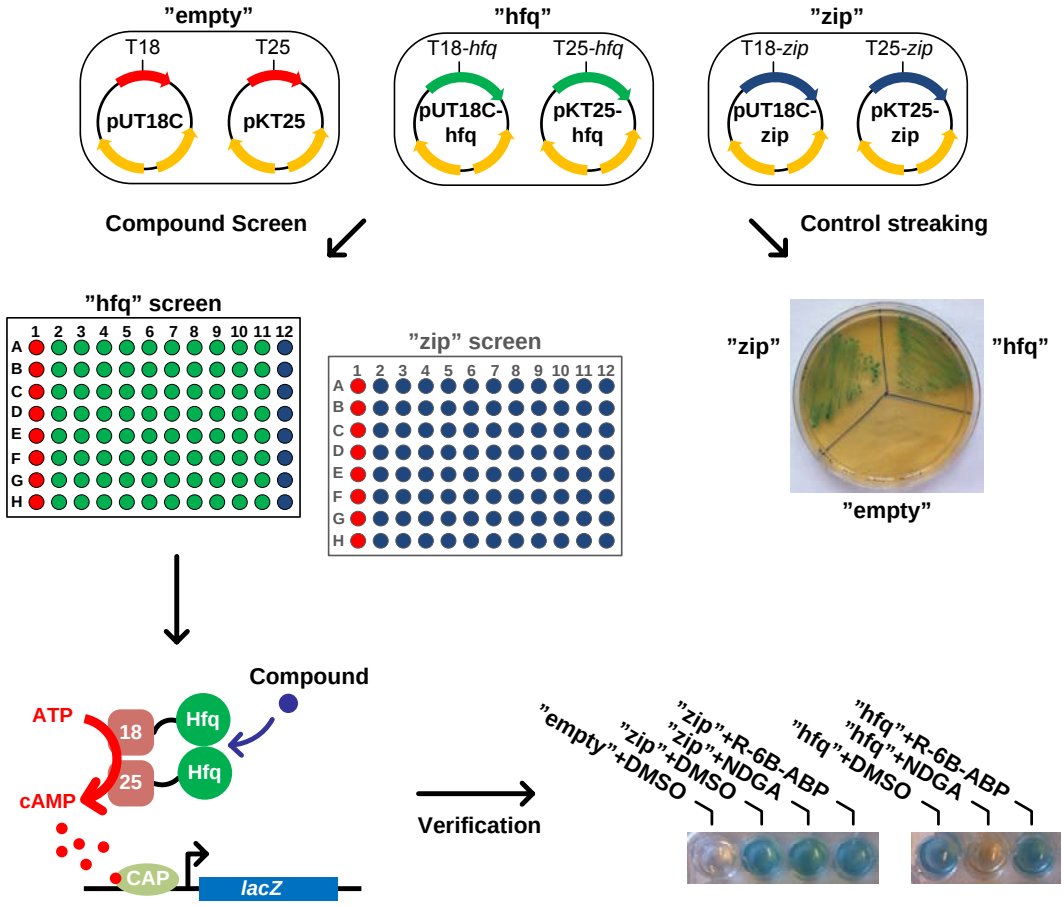

Figure 16. The bacterial two-hybrid screen as it was set up to select for inhibitors of Hfq-Hfq interaction. The assay was based on the restoration of adenylate cyclase activity in the *cya* mutant strain *E. coli* HB101. The control streaking verified that the Hfq interaction was as strong as the leucine zipper interaction. In order to exclude any false hits resulting from a general antimicrobial activity, we also included the 'zip screen' with the entire library. Protein-protein interaction is coupled to the growth of the reporter strain. Hence, negative and positive controls appear as blank or coloured wells, respectively. Compounds interfering with protein-protein interaction or exhibiting antimicrobial activity were indicated by reduced colour development. Manual verification of inhibitory activity of selected hits excluded the false positive entity R-6B-ABP but confirmed the Hfq-specific activity of NDGA.

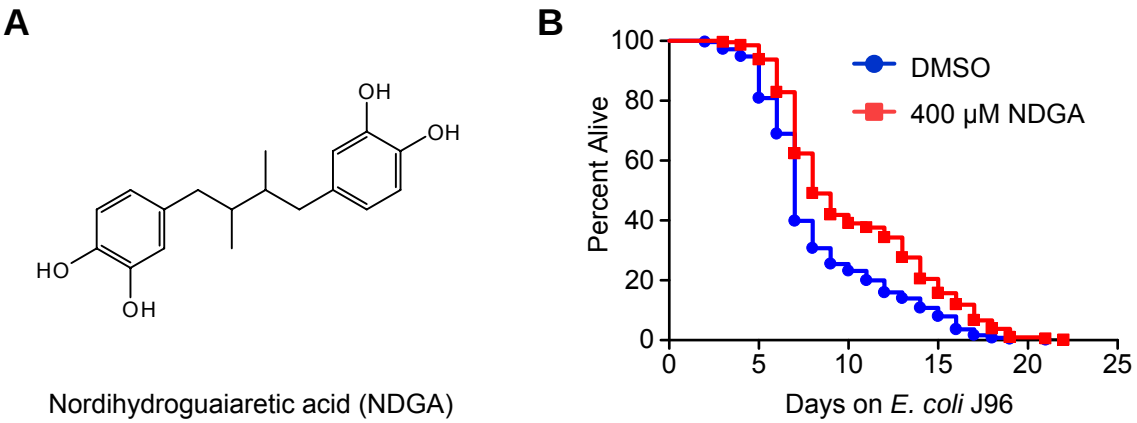

Figure 17. A: Chemical structure of nordihydroguaiaretic acid (NDGA). **B:** The lifespan of *C. elegans* AU37 feeding on *E. coli* J96, on 400 µM NDGA compared to the solvent DMSO.

Project 4 (see attached manuscript 2)

The alkaloid compound Harmane increases the lifespan of *Caenorhabditis elegans* during bacterial infection, by modulating the nematode's innate immune response.

The small molecule Harmane (2-methyl-β-carboline) was originally identified by Martin G. Marinus and Tao Xu at The University of Massachusetts Medical School. It was discovered in a 2-hybrid screen similar to the one described in project 3. The compound was found to interfere with the interaction between Int (intimin) and Tir (translocated intimin receptor) from the verotoxin producing *E. coli* EDL933 (Campellone and Leong 2003). See also figure 18. We were asked to test the compound in the *C. elegans* infection model. In project 2, we had seen that EDL933 was pathogenic towards the worm.

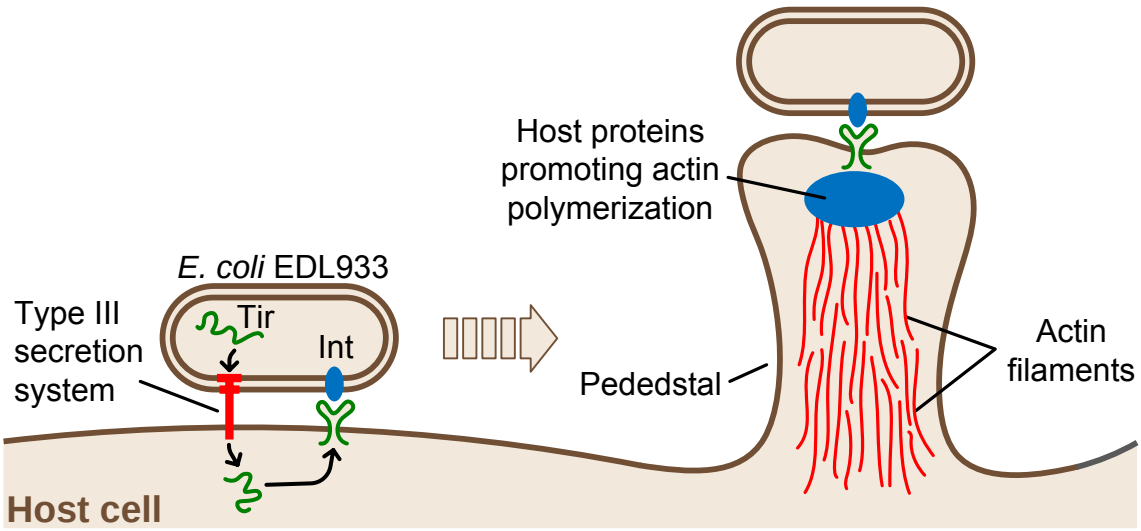

Figure 18. When *E. coli* EDL933 get in contact with the epithelial cell layer of the gut, it injects the Tir (translocated intimin receptor) into the cytoplasm of the epithelial cell using its type III secretion system. Tir then inserts itself into the apical plasma membrane of the cell where it acts as a receptor for the bacterial intimin protein. Once the interaction between Int and Tir is established, Tir recruits host cell proteins to form an actin pedestal under the bacterium. The pedestals destroy the microvilli of the epithelial cells, forming what are called attaching and effacing (AE) lesions. The figure was adapted from Alberts, 2008.

Results and discussion

We rather quickly learned that the Int-Tir interaction was not involved in the pathogenesis of *E. coli* EDL933 towards the nematode. However, we also discovered that Harmane significantly increased the lifespan of the worm from an LT50 of 7 days on the solvent DMSO to 17 days on 150 µM Harmane. These assays were performed in an immune deficient mutant worm: the mutant AU37 (*sek-1*; *glp*-4) is deficient in the p38 MAPK pathway. Initially we believed that the target was in the bacteria, but we soon turned our attention towards the innate immune system of the nematode. Further assays revealed that the effect of Harmane was most pronounced in this particular mutant worm. The effect of Harmane was less pronounced in worm which were deficient in the insulin-like receptor pathway and also in worms with an intact immune response (see figure 19).

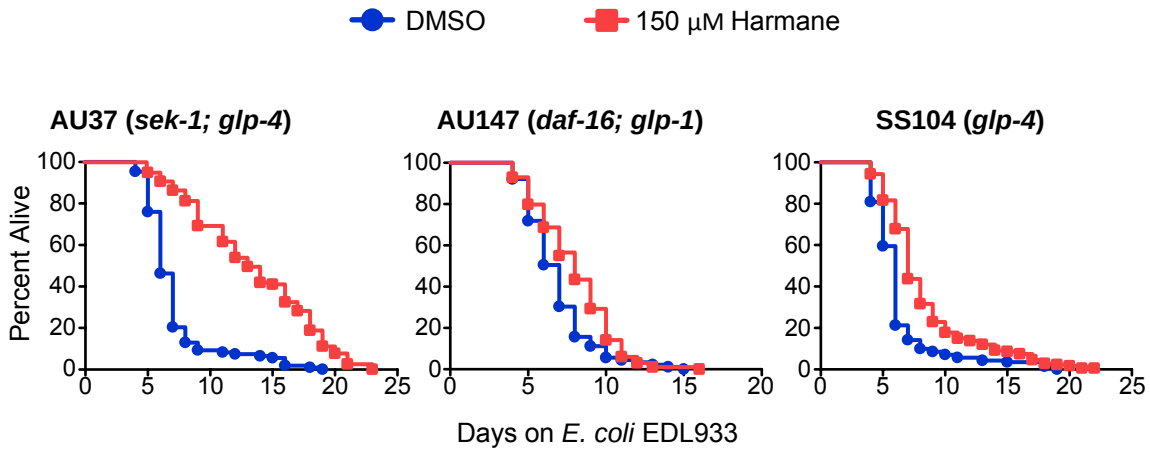

Figure 19. The effect of 150 µM Harmane versus the solvent DMSO in three different worm strains. *Sek-1* worms are deficient in the p38 MAPK signalling pathway. *Daf-16* nematodes are deficient in the insulin-like receptor pathway. SS104 worms have no defects in their immune pathways. The *glp* mutations (*glp-1* and *glp-4*) result in temperature sensitive sterility, this allows the worm to live on plates at 25°C without producing prodigy.

The fact that the effect of Harmane was most pronounced in a p38 MAPK deficient nematode suggested to us that Harmane activated one or more pathways parallel to the p38MAPK pathway and that they were not the insulin-like receptor pathway. We also speculate that these unknown parallel pathways become up-regulated when the p38 MAPK pathway is inactivated. This phenomenon of up-regulation of immune effector

genes, in order to compensate for the loss of similar genes, has been recently reported in *C. elegans* (Marsh *et al.* 2011). Figure 20 shows a model of how we believe this compensation to function.

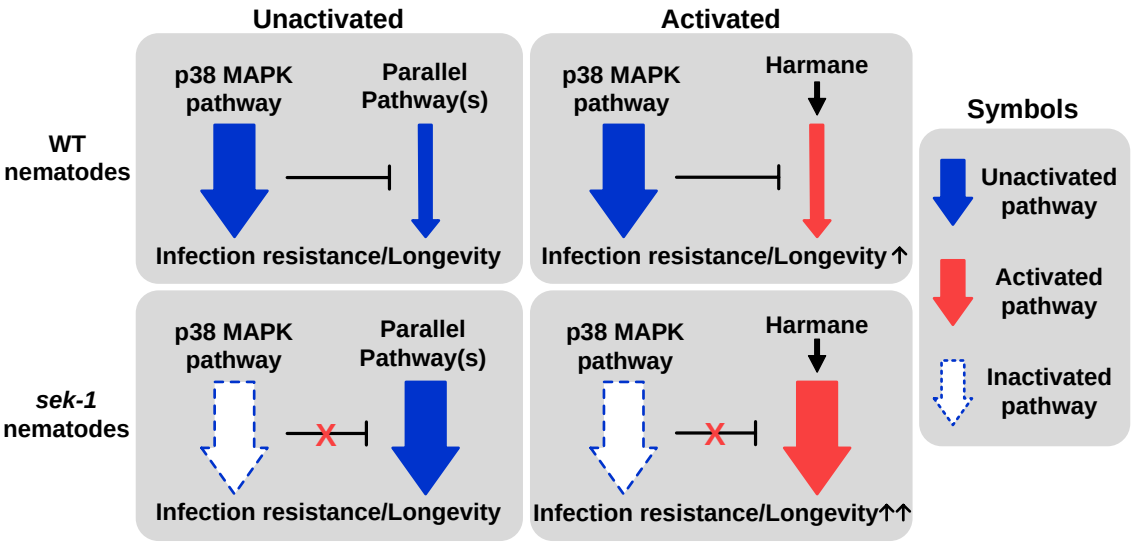

Figure 20. Hypothetical model for the Harmane induced pathway and its relation to the p38 MAPK pathway. We propose that Harmane acts on a pathway parallel to that of p38 MAPK pathway. This is likely not to be the insulin-like receptor pathway. We believe that in wild-type nematodes this pathway is held in check by the p38 MAPK pathway, resulting in only a minor activation by Harmane. We suggest that in the absence of the p38 MAPK pathway this parallel pathway is up-regulated in a compensatory fashion. This results in the increased pathogen resistance and extended longevity observed in *sek-1; glp-4* nematodes, when treated with Harmane, compared to nematodes with an intact p38 MAPK pathway.

Future perspectives

Of the 4 projects described in this thesis aimed at discovering new compounds targeting bacterial pathogens, only one resulted in a somewhat successful compound (Harmane). The success of this project was based on the pure luck by observing that Harmane affected survival of AU37 (*sek-1*; *glp-4*) nematodes – completely unrelated to the interaction we actually investigated. If we had used a nematode other than AU37, for example a wild-type worm as is custom when investigating immuno-modulation, we would probably not have observed any effect from Harmane. Harmane was selected to target the Intimin-Tir interaction, and the choice of the nematode strain was purely coincidental.

So I believe there are two conclusions that we can draw: a) it is better to be lucky than good, and b) it is very difficult to intelligently design a specific assay. Many choices have to be made, and perhaps one wrong/bad choice can ruin the final outcome. So is the lesson that we have to rely purely on luck? I believe that it would be beneficial to adopt a high-throughput approach to these assays: I believe that it would be beneficial to test more of the possible combinations when designing an assay. In project 1 for example we had the choice of using a *lac* promoter or the arabinose promoter, we chose the P_{BAD} promoter in order to be able to induce ToxT independent of the peptide library. Perhaps the *lac* operator would have been a better choice. In project 3 we could have tested both the C-terminal and N-terminal fusions between Hfq and CyaA. This would have cost more but it would perhaps have saved us many wasted man-hours on a failed screen.

References

Abuaita, B.H. and Withey, J.H. (2009) Bicarbonate Induces *Vibrio cholerae* virulence gene expression by enhancing ToxT activity. *Infection and immunity*, **77**, 4111-4120.

Alberts, B. (2008) *Molecular biology of the cell* 5th ed. edn. New York: Garland Science ; [London : Taylor & Francis, distributor].

Austin, D.J., Kristinsson, K.G. and Anderson, R.M. (1999) The relationship between the volume of antimicrobial consumption in human communities and the frequency of resistance. *Proceedings of the National Academy of Sciences of the United States of America*, **96**, 1152-1156.

Ball, P.R., Shales, S.W. and Chopra, I. (1980) Plasmid-mediated tetracycline resistance in *Escherichia coli* involves increased efflux of the antibiotic. *Biochemical and biophysical research communications*, **93**, 74-81.

Baylis, H.A. and Vazquez-Manrique, R.P. (2012) Genetic analysis of IP3 and calcium signalling pathways in *C. elegans*. *Biochimica et biophysica acta*, **1820**, 1253-1268.

Benveniste, R. and Davies, J. (1973) Aminoglycoside antibiotic-inactivating enzymes in actinomycetes similar to those present in clinical isolates of antibiotic-resistant bacteria. *Proceedings of the National Academy of Sciences of the United States of America*, **70**, 2276-2280.

Bolz, D.D., Tenor, J.L. and Aballay, A. (2010) A conserved PMK-1/p38 MAPK is required in *Caenorhabditis elegans* tissue-specific immune response to *Yersinia pestis* infection. *The Journal of biological chemistry*, **285**, 10832-10840.

Bowers, L.M., Lapoint, K., Anthony, L., Pluciennik, A. and Filutowicz, M. (2004) Bacterial expression system with tightly regulated gene expression and plasmid copy number. *Gene*, **340**, 11-18.

Breger, J., Fuchs, B.B., Aperis, G., Moy, T.I., Ausubel, F.M. and Mylonakis, E. (2007) Antifungal chemical compounds identified using a *C. elegans* pathogenicity assay. *PLoS pathogens*, **3**, e18.

Campellone, K.G. and Leong, J.M. (2003) Tails of two Tirs: actin pedestal formation by enteropathogenic *E. coli* and enterohemorrhagic *E. coli* O157:H7. *Current opinion in microbiology*, **6**, 82-90.

Cetinkaya, Y., Falk, P. and Mayhall, C.G. (2000) Vancomycin-resistant enterococci. *Clinical microbiology reviews*, **13**, 686-707.

Chao, Y. and Vogel, J. (2010) The role of Hfq in bacterial pathogens. *Current opinion in microbiology*, **13**, 24-33.

Clatworthy, A.E., Pierson, E. and Hung, D.T. (2007) Targeting virulence: a new paradigm for antimicrobial therapy. *Nat Chem Biol*, **3**, 541-548.

Davies, J. (1995) Vicious circles: looking back on resistance plasmids. *Genetics*, **139**, 1465-1468.

Davis, B.D. (1987) Mechanism of bactericidal action of aminoglycosides. *Microbiological reviews*, **51**, 341-350.

Davis, B.D. and Mingioli, E.S. (1950) Mutants of *Escherichia coli* requiring methionine or vitamin B12. *Journal of bacteriology*, **60**, 17-28.

Davis, B.M., Lawson, E.H., Sandkvist, M., Ali, A., Sozhamannan, S. and Waldor, M.K. (2000) Convergence of the secretory pathways for cholera toxin and the filamentous phage, CTXphi. *Science*, **288**, 333-335.

De Lay, N., Schu, D.J. and Gottesman, S. (2013) Bacterial Small RNA-based Negative Regulation: Hfq and Its Accomplices. *The Journal of biological chemistry*, **288**, 7996-8003.

Drlica, K., Malik, M., Kerns, R.J. and Zhao, X. (2008) Quinolone-mediated bacterial death. *Antimicrobial agents and chemotherapy*, **52**, 385-392.

Ehmann, D.E., Jahic, H., Ross, P.L., Gu, R.F., Hu, J., Kern, G., Walkup, G.K. and Fisher, S.L. (2012) Avibactam is a covalent, reversible, non-beta-lactam beta-lactamase inhibitor. *Proceedings of the National Academy of Sciences of the United States of America*, **109**, 11663-11668.

Evans, T.C., Jr., Martin, D., Kolly, R., Panne, D., Sun, L., Ghosh, I., Chen, L., Benner, J., Liu, X.Q. and Xu, M.Q. (2000) Protein trans-splicing and cyclization by a naturally split intein from the *dnaE* gene of *Synechocystis* species PCC6803. *The Journal of biological chemistry*, **275**, 9091-9094.

Franklin, T.J., Snow, G.A. and Franklin, T.J. (2005) *Biochemistry and molecular biology of antimicrobial drug action* 6th edn. New York: Springer.

French, G.L. (2010) The continuing crisis in antibiotic resistance. *Int J Antimicrob Agents*, **36 Suppl 3**, S3-7.

Hakenbeck, R. (2000) Transformation in *Streptococcus pneumoniae*: mosaic genes and the regulation of competence. *Research in microbiology*, **151**, 453-456.

Hancock, R.E., Nijnik, A. and Philpott, D.J. (2012) Modulating immunity as a therapy for bacterial infections. *Nature reviews*, **10**, 243-254.

Hooper, D.C. (2000) Mechanisms of action and resistance of older and newer fluoroquinolones. *Clin Infect Dis*, **31 Suppl 2**, S24-28.

Hope, I.A. (1999) *C. elegans : a practical approach* Oxford ; New York: Oxford University Press.

Hopwood, D.A. (2007) How do antibiotic-producing bacteria ensure their self-resistance before antibiotic biosynthesis incapacitates them? *Molecular microbiology*, **63**, 937-940.

Hung, D.L., Knight, S.D., Woods, R.M., Pinkner, J.S. and Hultgren, S.J. (1996) Molecular basis of two subfamilies of immunoglobulin-like chaperones. *The EMBO journal*, **15**, 3792-3805.

Hung, D.T. and Mekalanos, J.J. (2005) Bile acids induce cholera toxin expression in *Vibrio cholerae* in a ToxT-independent manner. *Proceedings of the National Academy of Sciences of the United States of America*, **102**, 3028-3033.

Hung, D.T., Shakhnovich, E.A., Pierson, E. and Mekalanos, J.J. (2005) Small-molecule inhibitor of *Vibrio cholerae* virulence and intestinal colonization. *Science*, **310**, 670-674.

Irazoqui, J.E., Troemel, E.R., Feinbaum, R.L., Luhachack, L.G., Cezairliyan, B.O. and Ausubel, F.M. (2010) Distinct pathogenesis and host responses during

infection of *C. elegans* by *P. aeruginosa* and *S. aureus*. *PLoS pathogens*, **6**, e1000982.

Jacoby, G.A. and Munoz-Price, L.S. (2005) The new beta-lactamases. *The New England journal of medicine*, **352**, 380-391.

Karimova, G., Pidoux, J., Ullmann, A. and Ladant, D. (1998) A bacterial two-hybrid system based on a reconstituted signal transduction pathway. *Proceedings of the National Academy of Sciences of the United States of America*, **95**, 5752-5756.

Kauppi, A.M., Nordfelth, R., Uvell, H., Wolf-Watz, H. and Elofsson, M. (2003) Targeting bacterial virulence: inhibitors of type III secretion in Yersinia. *Chemistry & biology*, **10**, 241-249.

Kohanski, M.A., Dwyer, D.J. and Collins, J.J. (2010) How antibiotics kill bacteria: from targets to networks. *Nature reviews*, **8**, 423-435.

Kohanski, M.A., Dwyer, D.J., Hayete, B., Lawrence, C.A. and Collins, J.J. (2007) A common mechanism of cellular death induced by bactericidal antibiotics. *Cell*, **130**, 797-810.

LeBlanc, J.J. (2003) Implication of virulence factors in *Escherichia coil* O157:H7 pathogenesis. *Critical reviews in microbiology*, **29**, 277-296.

Leclercq, R. and Courvalin, P. (1991) Bacterial resistance to macrolide, lincosamide, and streptogramin antibiotics by target modification. *Antimicrobial agents and chemotherapy*, **35**, 1267-1272.

Levy, S.B. and Marshall, B. (2004) Antibacterial resistance worldwide: causes, challenges and responses. *Nature medicine*, **10**, S122-129.

Long, K.S., Poehlsgaard, J., Kehrenberg, C., Schwarz, S. and Vester, B. (2006) The Cfr rRNA methyltransferase confers resistance to Phenicols, Lincosamides, Oxazolidinones, Pleuromutilins, and Streptogramin A antibiotics. *Antimicrobial agents and chemotherapy*, **50**, 2500-2505.

Lucchesi, P., Carraway, M. and Marinus, M.G. (1986) Analysis of forward mutations induced by *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine in the bacteriophage P22 *mnt* repressor gene. *Journal of bacteriology*, **166**, 34-37.

Magalhaes, J.G., Tattoli, I. and Girardin, S.E. (2007) The intestinal epithelial barrier: how to distinguish between the microbial flora and pathogens. *Seminars in immunology*, **19**, 106-115.

Malhotra-Kumar, S., Lammens, C., Coenen, S., Van Herck, K. and Goossens, H. (2007) Effect of azithromycin and clarithromycin therapy on pharyngeal carriage of macrolide-resistant streptococci in healthy volunteers: a randomised, double-blind, placebo-controlled study. *Lancet*, **369**, 482-490.

Mariam, D.H., Mengistu, Y., Hoffner, S.E. and Andersson, D.I. (2004) Effect of *rpoB* mutations conferring rifampin resistance on fitness of *Mycobacterium tuberculosis*. *Antimicrobial agents and chemotherapy*, **48**, 1289-1294.

Marsh, E.K., van den Berg, M.C. and May, R.C. (2011) A two-gene balance regulates *Salmonella* Typhimurium tolerance in the nematode *Caenorhabditis elegans*. *PloS one*, **6**, e16839.

Medzhitov, R. (2009) Approaching the asymptote: 20 years later. *Immunity*, **30**, 766-775.

Miller, M.B. and Bassler, B.L. (2001) Quorum sensing in bacteria. *Annual review of microbiology*, **55**, 165-199.

Moy, T.I., Ball, A.R., Anklesaria, Z., Casadei, G., Lewis, K. and Ausubel, F.M. (2006) Identification of novel antimicrobials using a live-animal infection model. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 10414-10419.

Muh, U., Schuster, M., Heim, R., Singh, A., Olson, E.R. and Greenberg, E.P. (2006) Novel Pseudomonas aeruginosa quorum-sensing inhibitors identified in an ultra-high-throughput screen. *Antimicrobial agents and chemotherapy*, **50**, 3674-3679.

Murray, I.A. and Shaw, W.V. (1997) O-Acetyltransferases for chloramphenicol and other natural products. *Antimicrobial agents and chemotherapy*, **41**, 1-6.

Nijnik, A., Madera, L., Ma, S., Waldbrook, M., Elliott, M.R., Easton, D.M., Mayer, M.L., Mullaly, S.C., Kindrachuk, J., Jenssen, H. and Hancock, R.E. (2010) Synthetic cationic peptide IDR-1002 provides protection against bacterial infections through chemokine induction and enhanced leukocyte recruitment. *J Immunol*, **184**, 2539-2550.

Nikaido, H. (1994) Prevention of drug access to bacterial targets: permeability barriers and active efflux. *Science*, **264**, 382-388.

Nordfelth, R., Kauppi, A.M., Norberg, H.A., Wolf-Watz, H. and Elofsson, M. (2005) Small-molecule inhibitors specifically targeting type III secretion. *Infection and immunity*, **73**, 3104-3114.

Olsen, A.S., Moller-Jensen, J., Brennan, R.G. and Valentin-Hansen, P. (2010) C-terminally truncated derivatives of *Escherichia coli* Hfq are proficient in riboregulation. *Journal of molecular biology*, **404**, 173-182.

Patel, U., Yan, Y.P., Hobbs, F.W., Jr., Kaczmarczyk, J., Slee, A.M., Pompliano, D.L., Kurilla, M.G. and Bobkova, E.V. (2001) Oxazolidinones mechanism of action: inhibition of the first peptide bond formation. *The Journal of biological chemistry*, **276**, 37199-37205.

Piddock, L.J. (2006) Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. *Clinical microbiology reviews*, **19**, 382-402.

Pinkner, J.S., Remaut, H., Buelens, F., Miller, E., Aberg, V., Pemberton, N., Hedenstrom, M., Larsson, A., Seed, P., Waksman, G., Hultgren, S.J. and Almqvist, F. (2006) Rationally designed small compounds inhibit pilus biogenesis in uropathogenic bacteria. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 17897-17902.

Prouty, M.G., Osorio, C.R. and Klose, K.E. (2005) Characterization of functional domains of the *Vibrio cholerae* virulence regulator ToxT. *Molecular microbiology*, **58**, 1143-1156.

Prudhomme, M., Attaiech, L., Sanchez, G., Martin, B. and Claverys, J.P. (2006) Antibiotic stress induces genetic transformability in the human pathogen *Streptococcus pneumoniae*. *Science*, **313**, 89-92.

Pukkila-Worley, R., Feinbaum, R., Kirienko, N.V., Larkins-Ford, J., Conery, A.L. and Ausubel, F.M. (2012) Stimulation of Host Immune Defenses by a Small

Molecule Protects *C. elegans* from Bacterial Infection. *PLoS genetics*, **8**, e1002733.

Sack, D.A., Sack, R.B., Nair, G.B. and Siddique, A.K. (2004) Cholera. *Lancet*, **363**, 223-233.

Sauer, F.G., Barnhart, M., Choudhury, D., Knight, S.D., Waksman, G. and Hultgren, S.J. (2000) Chaperone-assisted pilus assembly and bacterial attachment. *Current opinion in structural biology*, **10**, 548-556.

Sauer, F.G., Remaut, H., Hultgren, S.J. and Waksman, G. (2004) Fiber assembly by the chaperone-usher pathway. *Biochimica et biophysica acta*, **1694**, 259-267.

Schulenburg, H., Kurz, C.L. and Ewbank, J.J. (2004) Evolution of the innate immune system: the worm perspective. *Immunol Rev*, **198**, 36-58.

Scott, C.P., Abel-Santos, E., Wall, M., Wahnon, D.C. and Benkovic, S.J. (1999) Production of cyclic peptides and proteins *in vivo*. *Proceedings of the National Academy of Sciences of the United States of America*, **96**, 13638-13643.

Scott, M.G., Dullaghan, E., Mookherjee, N., Glavas, N., Waldbrook, M., Thompson, A., Wang, A., Lee, K., Doria, S., Hamill, P., Yu, J.J., Li, Y., Donini, O., Guarna, M.M., Finlay, B.B., North, J.R. and Hancock, R.E. (2007) An anti-infective peptide that selectively modulates the innate immune response. *Nature biotechnology*, **25**, 465-472.

Shakhnovich, E.A., Hung, D.T., Pierson, E., Lee, K. and Mekalanos, J.J. (2007) Virstatin inhibits dimerization of the transcriptional activator ToxT. *Proceedings of the National Academy of Sciences of the United States of America*, **104**, 2372-2377.

Shinabarger, D.L., Marotti, K.R., Murray, R.W., Lin, A.H., Melchior, E.P., Swaney, S.M., Dunyak, D.S., Demyan, W.F. and Buysse, J.M. (1997) Mechanism of action of oxazolidinones: effects of linezolid and eperezolid on translation reactions. *Antimicrobial agents and chemotherapy*, **41**, 2132-2136.

Shivers, R.P., Youngman, M.J. and Kim, D.H. (2008) Transcriptional responses to pathogens in *Caenorhabditis elegans*. *Current opinion in microbiology*, **11**, 251-256.

Shoemaker, N.B., Vlamakis, H., Hayes, K. and Salyers, A.A. (2001) Evidence for extensive resistance gene transfer among Bacteroides spp. and among Bacteroides and other genera in the human colon. *Applied and environmental microbiology*, **67**, 561-568.

Siegele, D.A. and Hu, J.C. (1997) Gene expression from plasmids containing the *araBAD* promoter at subsaturating inducer concentrations represents mixed populations. *Proceedings of the National Academy of Sciences of the United States of America*, **94**, 8168-8172.

Sifri, C.D., Begun, J. and Ausubel, F.M. (2005) The worm has turned--microbial virulence modeled in *Caenorhabditis elegans*. *Trends in microbiology*, **13**, 119-127.

Skurray, R.A. and Firth, N. (1997) Molecular evolution of multiply-antibiotic-resistant staphylococci. *Ciba Foundation symposium*, **207**, 167-183; discussion 183-191.

Smith, K.M., Bu, Y. and Suga, H. (2003) Induction and inhibition of Pseudomonas aeruginosa quorum sensing by synthetic autoinducer analogs. *Chemistry & biology*, **10**, 81-89.

Sreevatsan, S., Pan, X., Stockbauer, K.E., Williams, D.L., Kreiswirth, B.N. and Musser, J.M. (1996) Characterization of *rpsL* and *rrs* mutations in streptomycin-resistant *Mycobacterium tuberculosis* isolates from diverse geographic localities. *Antimicrobial agents and chemotherapy*, **40**, 1024-1026.

Svensson, A., Larsson, A., Emtenas, H., Hedenstrom, M., Fex, T., Hultgren, S.J., Pinkner, J.S., Almqvist, F. and Kihlberg, J. (2001) Design and evaluation of pilicides: potential novel antibacterial agents directed against uropathogenic Escherichia coli. *Chembiochem*, **2**, 915-918.

Tavassoli, A. and Benkovic, S.J. (2007) Split-intein mediated circular ligation used in the synthesis of cyclic peptide libraries in *E. coli*. *Nature protocols*, **2**, 1126-1133.

Theuretzbacher, U. (2012) Accelerating resistance, inadequate antibacterial drug pipelines and international responses. *Int J Antimicrob Agents*, **39**, 295-299.

Tomasz, A. (1979) The mechanism of the irreversible antimicrobial effects of penicillins: how the beta-lactam antibiotics kill and lyse bacteria. *Annual review of microbiology*, **33**, 113-137.

Trieu-Cuot, P., Derlot, E. and Courvalin, P. (1993) Enhanced conjugative transfer of plasmid DNA from *Escherichia coli* to *Staphylococcus aureus* and *Listeria monocytogenes*. *FEMS microbiology letters*, **109**, 19-23.

Troemel, E.R., Chu, S.W., Reinke, V., Lee, S.S., Ausubel, F.M. and Kim, D.H. (2006) p38 MAPK regulates expression of immune response genes and contributes to longevity in *C. elegans*. *PLoS genetics*, **2**, e183.

Tsiodras, S., Gold, H.S., Sakoulas, G., Eliopoulos, G.M., Wennersten, C., Venkataraman, L., Moellering, R.C. and Ferraro, M.J. (2001) Linezolid resistance in a clinical isolate of *Staphylococcus aureus*. *Lancet*, **358**, 207-208.

Vaitkevicius, K., Lindmark, B., Ou, G., Song, T., Toma, C., Iwanaga, M., Zhu, J., Andersson, A., Hammarstrom, M.L., Tuck, S. and Wai, S.N. (2006) A *Vibrio cholerae* protease needed for killing of *Caenorhabditis elegans* has a role in protection from natural predator grazing. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 9280-9285.

Vecerek, B., Rajkowitsch, L., Sonnleitner, E., Schroeder, R. and Blasi, U. (2008) The C-terminal domain of *Escherichia coli* Hfq is required for regulation. *Nucleic acids research*, **36**, 133-143.

Vogel, J. and Luisi, B.F. (2011) Hfq and its constellation of RNA. *Nature reviews*, **9**, 578-589.

Wilson, J.W., Ott, C.M., Honer zu Bentrup, K., Ramamurthy, R., Quick, L., Porwollik, S., Cheng, P., McClelland, M., Tsaprailis, G., Radabaugh, T., Hunt, A., Fernandez, D., Richter, E., Shah, M., Kilcoyne, M., Joshi, L., Nelman-Gonzalez, M., Hing, S., Parra, M., Dumars, P., Norwood, K., Bober, R., Devich, J., Ruggles, A., Goulart, C., Rupert, M., Stodieck, L., Stafford, P., Catella, L., Schurr, M.J., Buchanan, K., Morici, L.,

McCracken, J., Allen, P., Baker-Coleman, C., Hammond, T., Vogel, J., Nelson, R., Pierson, D.L., Stefanyshyn-Piper, H.M. and Nickerson, C.A. (2007) Space flight alters bacterial gene expression and virulence and reveals a role for global regulator Hfq. *Proceedings of the National Academy of Sciences of the United States of America*, **104**, 16299-16304.

Wright, G.D., Berghuis, A.M. and Mobashery, S. (1998) Aminoglycoside antibiotics. Structures, functions, and resistance. *Advances in experimental medicine and biology*, **456**, 27-69.

Wu, H., Hu, Z. and Liu, X.Q. (1998) Protein trans-splicing by a split intein encoded in a split *DnaE* gene of *Synechocystis* sp. PCC6803. *Proceedings of the National Academy of Sciences of the United States of America*, **95**, 9226-9231.

Yoneyama, H., Yamano, Y. and Nakae, T. (1995) Role of porins in the antibiotic susceptibility of *Pseudomonas aeruginosa*: construction of mutants with deletions in the multiple porin genes. *Biochemical and biophysical research communications*, **213**, 88-95.

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Short communication

Lack of the RNA chaperone Hfq attenuates pathogenicity of several *Escherichia coli* pathotypes towards *Caenorhabditis elegans*

Martin S. Bojer [a,b,1], Henrik Jakobsen [a,b,1], Carsten Struve [b], Karen A. Krogfelt [b], Anders Løbner-Olesen [a,c,*]

[a] *Department of Science, Systems and Models, Roskilde University, Universitetsvej 1, DK-4000 Roskilde, Denmark*
[b] *Department of Microbiology and Infection Control, Statens Serum Institut, Artillerivej 5, DK-2300 Copenhagen S, Denmark*
[c] *Department of Biology, University of Copenhagen, Ole Maaløes Vej 5, DK-2200 Copenhagen N, Denmark*

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Abstract

Escherichia coli is an important agent of Gram-negative bacterial infections worldwide, being one of the leading causes of diarrhoea and urinary tract infections. Strategies to understand pathogenesis and develop therapeutic compounds include the use of the nematode *Caenorhabditis elegans* as a model for virulence characterization and screening for novel antimicrobial entities. Several *E. coli* human pathotypes are also pathogenic towards *C. elegans*, and we show here that lack of the RNA chaperone Hfq significantly reduces pathogenicity of VTEC, EAEC, and UPEC in the nematode model. Thus, Hfq is intrinsically essential to pathogenic *E. coli* for survival and virulence exerted in the *C. elegans* host.

Keywords: *E. coli*; Virulence; Hfq; *C. elegans*; Antivirulence; In vivo model

1. Introduction

The enterobacterium *Escherichia coli* is a commensal of the human gastrointestinal tract. However, it is also a versatile pathogen causing intestinal and extraintestinal diseases in immunocompromised and healthy individuals. The wide spectrum of clinical symptoms attributable to *E. coli* is a reflection of the genetic variability within this species. Thus, disease-causing *E. coli* are generally divided into different pathotypes. The diarrhoeagenic *E. coli* constitutes a major group of several pathotypes encompassing e.g. Vero cytotoxin-producing *E. coli* (VTEC) responsible for acute and (often) bloody diarrhoea associated with the severe clinical manifestation haemolytic uremic syndrome (HUS), and enteroaggregative *E. coli* (EAEC) giving rise to acute as well as persistent diarrhoea mainly among inhabitants of developing countries but also in travellers [1]. Uropathogenic *E. coli* (UPEC), a pathotype within the extraintestinal pathogenic *E. coli* (ExPEC), is probably the most common agent of human urinary tract infections [1]. Each pathotype is described by histopathological and phenotypic appearances as well as more or less specific combinations of virulence genes. Given the high morbidity, and greatly spurred by the worldwide development of antibiotic resistance, further studies on virulence mechanisms and identification of new antimicrobial targets in *E. coli* are warranted.

A relatively novel approach in antimicrobial development is antivirulence therapy where factor(s) contributing to the pathogenesis of an infectious agent is the target of inhibition [2]. Targeting virulence would disarm a bacterial pathogen rather than kill it, which, in turn, may lower the selective pressure for evolution of resistance. The identification of an

* Corresponding author. Department of Science, Systems and Models, Roskilde University, Universitetsvej 1, DK-4000 Roskilde, Denmark. Tel.: +45 46742615; fax: +45 46743011.
E-mail address: lobner@ruc.dk (A. Løbner-Olesen).
[1] These authors contributed equally.

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inhibitor of a central virulence regulator in *Vibrio cholerae* constitutes an elegant example of an antivirulence compound [3]. Identification and development of antivirulence compounds require sophisticated in vitro setups or relevant in vivo models due to the non-bacteriocidal nature of such entities. Because many biological pathways are conserved between *Caenorhabditis elegans* and humans, the nematode is being increasingly used as a simple model organism to study virulence mechanisms of both bacterial and fungal infections [4]. Due to its simplicity and ease of cultivation, it is also an organism feasible for large screenings of antimicrobials, antivirulence compounds, and other factors promoting host survival following infection [5].

The pleiotropic role played by the RNA chaperone and posttranscriptional regulator Hfq in diverse bacterial pathogens is increasingly acknowledged [6]. Though generally viable, bacterial *hfq* mutants often display a lowered stress tolerance and attenuation in animal models. Thus, the broad implication of Hfq in global gene expression and virulence has been demonstrated for bacterial pathogens as diverse as *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Listeria monocytogenes* [7–9]. In the *C. elegans* infection model, Hfq was shown to be involved in virulence of *Burkholderia cepacia* and *Serratia* sp. [10,11]. A role for Hfq in multidrug resistance in *E. coli* has been described [12] adding further to the clinical significance of its regulon. Though vast amounts of biological functions of Hfq have been dissected in *E. coli*, its relevance in virulence of pathogenic *E. coli* remains less well described. One study, though, revealed a prominent role played by Hfq in fitness and virulence properties of uropathogenic *E. coli* [13]. Moreover, Hfq has also been shown to both positively and negatively affect the type III secretion systems, including effector proteins responsible for AE lesions and the hallmark pedestal formation phenotype, in different VTEC strains [14,15]. Thus, specific virulence properties may be differently integrated into the Hfq regulon even in strains of the same pathotype. Transcriptome arrays show that the Hfq regulon include *E. coli* core genome genes as well as, to a large degree, pathotype-specific genes [14]. Recently, involvement of Hfq in pathogenesis of adherent-invasive *E. coli* (AIEC) towards *C. elegans* was reported [16]. Here we tested the hypothesis that Hfq might be relevant for pathogenesis in *C. elegans* of disease-causing *E. coli* in general.

2. Materials and methods

2.1. Strains, plasmids and DNA manipulations

The nematode strain *C. elegans* AU37 [17], a derivative of Bristol N2 carrying the mutated *sek-1* and *glp-4* alleles (MAPK kinase deficiency and temperature-sensitive sterile), were maintained on NGM media [18] supplied with the food source *E. coli* OP50 at 15 °C.

Strains of *E. coli* and their derivatives, plasmids and primers used in this study are listed in Table 1. Strains were grown overnight at 37 °C with antibiotics when appropriate at the following concentrations: ampicillin 100 μg/ml, kanamycin 50 μg/ml, chloramphenicol 12.5 μg/ml, apramycin 30 μg/ml. PCR was performed using the Expand High Fidelity PCR System (Roche). The *hfq* gene including its upstream promoter region was amplified from *E. coli* MG1655 (NCBI gene id 948689) using primer pair F_hfq/R_hfq and was cloned (Fast-Link DNA Ligation Kit, Epicentre) into pBR322 by directional cloning using BamHI and SalI restriction enzymes (Fermentas). Plasmid pBAD18-GFP was constructed by amplification of the GFPmut2 gene [19] from pGFPmut2 with primer pair F-GFPmut2/R-GFPmut2 followed by insertion into sites EcoRI and SmaI in pBAD18. The linear *hfq*

Table 1
Bacterial strains, plasmids and primers used in this study.

Strains, plasmids, and primers	Description/sequence	Source or reference
Escherichia coli strains		
OP50	Standard food source/negative control for *C. elegans*	[18]
MG1655	WT K12 isolate	Lab. stock
17-2	EAEC prototype strain	Lab. stock
17-2Δ*hfq*	Strain carrying chromosomal *hfq*-deletion (Kn^R)	This study
EDL933	VTEC prototype strain	Lab. stock
EDL933Δ*hfq*	Strain carrying chromosomal *hfq*-deletion (Cam^R)	This study
J96	UPEC	Lab. stock
J96Δ*hfq*	Strain carrying chromosomal *hfq*-deletion (Kn^R)	This study
Plasmids		
pKOBEGapra	Plasmid encoding arabinose-inducible λ-red functions ($Apra^R$)	J.M. Ghigo
pGFPmut2	Plasmid containing gene encoding GFPmut2	[19]
phfq	pBR322 encoding *hfq* under its native promoter	This study
pBAD18-GFP	Plasmid expressing GFPmut2 from the P_{BAD} promoter	This study
Primers		
F-hfq	5′-gatacaggatcccatggctaaggggcaatc-3′	This study
R-hfq	5′-gatacagtcgacgaacgcaggatcgctggctc-3′	This study
F-Kn_hfq	5′-atggctaaggggcaatctttacaagatccgttaaagccacgttgtgtctc-3′	This study
R-Kn_hfq	5′-ttattcggtttcttcgctgtcctgttgcgcgggtcagcgtaatgctctgc-3′	This study
F-GFPmut2	5′-gcatggaattcgaaggagatatacatatgag-3′	This study
R-GFPmut2	5′-gctaccccgggcagttatttgtatagttcatcc-3′	This study

kanamycin deletion fragment was synthesized by PCR using primer pair F-Kn_hfq/R-Kn_hfq and plasmid pACYC177 as template. Transfer of genetic material was performed by standard electroporation.

2.2. Mutant constructions

The *hfq* gene in *E. coli* 17-2 was deleted by allelic exchange with the PCR synthesized cassette encoding kanamycin resistance flanked by regions homologous to sequences up- and down-stream of the *hfq* gene by the λ-Red mediated recombination procedure [20]. Allelic replacement was facilitated by the thermo-sensitive helper plasmid pKOBEGApra, an apramycin resistant derivative of pKOBEG [20], encoding lambda Red recombinase functions. *E. coli* 17-2 bearing plasmid pKOBEGApra were grown at 30 °C and induced by addition of 0.2% arabinose (Sigma). The purified PCR product was introduced to electro-competent 17-2 harbouring pKOBEGApra followed by temperature-induced (37 °C) curing of pKOBEGApra and kanamycin selection. The *hfq* deletion allele was subsequently transferred to *E. coli* J96 by amplification of the deleted gene using primers F-hfq and R-hfq and repeated λ-Red mediated recombination. The *hfq* deletion in *E. coli* EDL933 was obtained by transfer of a chloramphenicol-marked *hfq* deletion from a phage-negative EDL933 variant (M.G. Marinus). Correct allelic replacements were verified by PCR.

2.3. Growth and stress resistance assays

Growth kinetics of wild type and *hfq* mutants were evaluated by dilution of overnight cultures into fresh NGM media followed by incubation at 37 °C with rigorous shaking and manually recording OD_{450} at indicated time points. Sensitivity to oxidative stress was assessed by spotting ten-fold dilutions of exponential phase cultures normalized according to OD_{600} on LB-agar supplemented with 1.25 mM H_2O_2. Plates were incubated overnight at 37 °C.

2.4. C. elegans *killing assay*

A synchronous population of worms was obtained by releasing worm embryos by an alkaline hypochlorite treatment [18] followed by hatching in M9 buffer at 15 °C overnight. L1 larvae were transferred to NGM plates seeded with OP50 and allowed to develop into adult/L4 larvae for two days at 25°C, after which they were washed three times in M9 buffer and transferred to assay plates. Assay plates were prepared by seeding NGM plates with 20 μl overnight cultures of OP50 or test strains followed by overnight incubation at 37 °C. After acclimatization of the plates to room temperature at least 30 worms were transferred to each plate. Plates were incubated at 25 °C and scored for dead worms each day. A worm was considered dead when it failed to respond to a touch with a platinum wire; dead worms stuck to the wall of the plate were censored from analysis. Killing data from duplicate plates were pooled and subjected to survival analysis. Results presented are representative of repeated independent assays.

2.5. Visualization and quantification of bacterial intestinal colonization

C. elegans were fed on strains containing pBAD18-GFP on NGM plates containing ampicillin and 0.2% arabinose to activate expression of GFP from the P_{BAD} promoter. On day 4 the worms were washed from the plates into M9 media followed by 3 further washes to remove external bacteria. Subsequently, the worms were anaesthetized and immobilized by addition of 1% NaN_3 and placed on top of a 1.5% agarose pad on a microscope slide overlaid with a cover slide. Worms were examined and photographed with an Olympus BX61 microscope and an Olympus DP71 camera using the cell^P software (Olympus). All photographs were acquired using the same settings and a fixed exposure time of 50 ms. Quantitative assessment of bacterial colonization was done by determination of fluorescence intensity of at least 20 worms feeding on each bacterial strain by analysis of individual worms using ImageJ v1.45.

2.6. Statistics

Analysis of data was performed in GraphPad Prism version 5.00 (www.graphpad.com). Kaplan–Meier survival plots and log-rank test were used to analyse *C. elegans* killing data. Mean fluorescence of worms feeding on wild type or mutant strains ($n \geq 20$, each) were compared by non-parametric *t*-test (Mann–Whitney). Values of $P \leq 0.05$ were considered statistically significant.

3. Results and discussion

AIEC strain LF82 was recently shown to cause a lethal infection of *C. elegans* independent of several known virulence genes established for this pathotype. Nematode killing was, however, shown to depend strongly on the RNA chaperone Hfq [16]. We suspected that this phenomenon might not be confined to AIEC alone and sought to assess the necessity of Hfq for pathogenic *E. coli* more generally. Thus, prototype strains from the three major pathotypes VTEC (EDL933), EAEC (17-2), and UPEC (J96) were evaluated in the nematode assay and compared to their isogenic *hfq* deletion mutants. In agreement with previous reports using different experimental setups and nematode strains, we find that all three pathotypes are significantly more lethal to *C. elegans* than the apathogenic *E. coli* OP50 [21–23]. LT50 (time at which 50 percent of the population is dead) was 6 days for EDL933 and 17-2, but 8 days for J96, compared to 10 days for OP50 ($P < 0.001$) (Fig. 1). As depicted, deletion of *hfq* in every pathotype cause a significant reduction in pathogenicity for nematodes. The increase in LT50 due to lack of Hfq was 3, 4 and 7 days for strain EDL933, 17-2 and J96, respectively. In any case, the nematode killing phenotype was fully restored by complementation in trans (Fig. 1). Thus, though strain dependent, *hfq* is required for pathogenicity of any *E. coli*

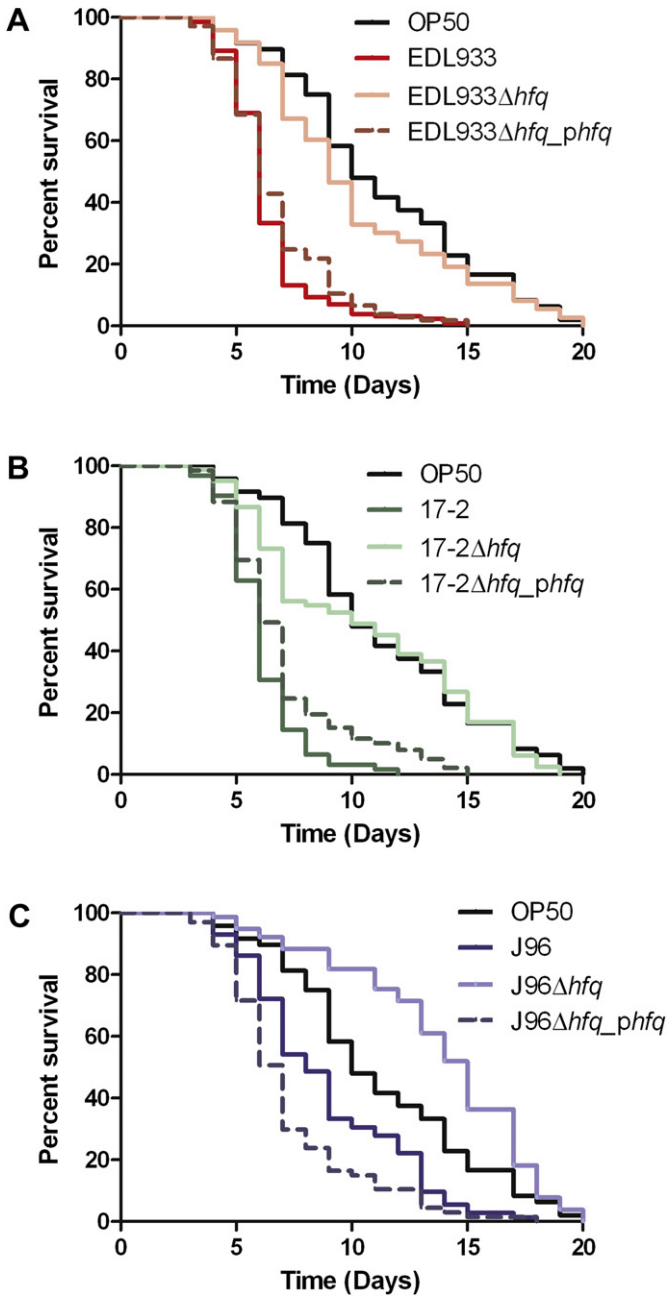

Fig. 1. Kaplan–Meier survival plots from *C. elegans* feeding on (A) VTEC (EDL933), (B) EAEC (17-2), and (C) UPEC (J96) strains, mutants lacking *hfq* and derivatives complemented in trans. *E. coli* OP50 included as a control. OP50, WT and mutant strains all carry the empty vector background (pBR322).

pathotype (assayed here) against *C. elegans*, and the severe reduction in virulence potential of strain J96 suggests that Hfq function is of paramount importance in in vivo fitness or virulence of UPEC strains. A strong correlation between mouse lethality and virulence of different extraintestinal pathogenic *E. coli* isolates in the nematode model has been reported [21], suggesting that *C. elegans* pathogenesis can provide a useful reflection of the virulence potential in a mammalian host. The fact that uropathogenesis of *E. coli* in mice is dependent on Hfq [13] further supports the use of the nematode for studies on bacterial virulence.

It is well established that deletion of *hfq* in *E. coli* cause pleiotrophic phenotypes in vitro including sub-optimal growth kinetics when grown in culture [24]. In AIEC, the lack of Hfq was reported to severely hamper nematode intestinal colonization [16]; a consequence also observed for *hfq* deletion mutants of *B. cepacia* and *Serratia* sp. [10,11]. Thus, the slow killing of *C. elegans* by *hfq* mutants is not necessarily attributable to the lack of expression of specific virulence factor(s) per se, but it may also result from delayed or absent colonization of the nematode. To address this question, we analysed the colonization burden in nematodes infected by all three pathotypes and their *hfq* negative counterparts used in the killing assay (Fig. 2). A general tendency to a lowered intestinal colonization at day 4 was evident for *hfq* deletion mutants of VTEC, EAEC as well as UPEC. Attenuation in colonization ability due to lacking Hfq was, however, only significant for UPEC under the conditions tested ($P < 0.01$) (Fig. 2A) giving rise to a clearly visible difference between nematodes infected with wild type and mutant bacteria (Fig. 2B). It is noteworthy that the relative colonization ability of the different *hfq* mutants inversely correlates with the increase in LT50 observed for the respective strains. Thus, nematode killing by pathogenic *E. coli* most likely results directly from intestinal overgrowth. We do observe a slightly extended lag-phase when recovered from stationary phase and a lowered stationary phase cell density for all three *hfq* mutants when grown in culture. This phenotype is exacerbated in low-nutrient media such as NGM (Fig. 2C); a phenotype previously reported for an EDL933 *hfq* mutant in other minimal media [15]. Therefore, loss of nematode infectivity may result from the physiological fitness cost due to lack of Hfq. The fact that EDL933 is almost as severely impaired in growth as J96 when devoid of Hfq (Fig. 2C) suggests that growth kinetics are not the sole explanation for the observed nematode killing rate, however. The fitness cost is not necessarily confined to growth rate, but it may also stem from a lower tolerance to stresses encountered in the nematode intestine as proposed for *Serratia* sp. [10]. As an example, we notice an increased sensitivity of all mutants to oxidative stress (Fig. 2D), a phenotype also demonstrated for the other Gram-negative Hfq-dependent *C. elegans* pathogens [10,11,16]. In this assay, the degree of stress sensitivity of the different *hfq* mutants inversely correlates with the killing rate observed in the nematode. Collectively, our data, and the data presented on AIEC [16], suggest that Hfq is indispensible for pathogenicity of *E. coli* in general. Thus, species-related effects, rather than pathotype-specific effects, are probably the cause of an attenuated pathogenicity towards *C. elegans*. This indispensability is, however, pathotype or even strain specific, and, in the set of strains tested here, Hfq was most essential for general fitness of the UPEC strain. Hfq is known to affect virulence attributes, such as motility, biofilm formation, resistance to antimicrobial peptides, and intracellular behaviour in UPEC strain UTI89 [13]. Due to the significant and severe reduction in pathogenesis towards *C. elegans* demonstrated for UPEC strain J96, we cannot exclude the possibility that more specific UPEC virulence traits, also

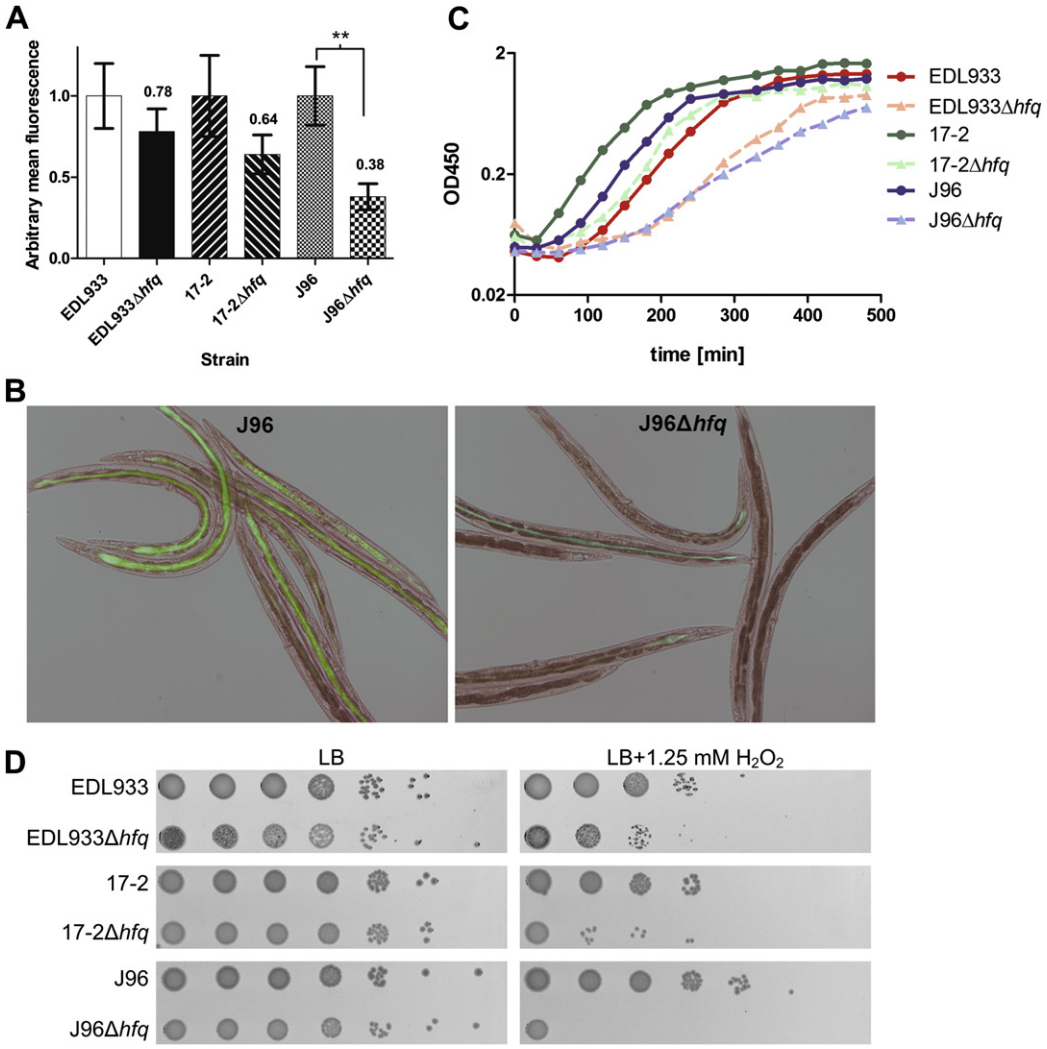

Fig. 2. (A) Assessment of necessity of Hfq for nematode intestinal colonization of VTEC (EDL933), EAEC (17-2) and UPEC (J96) strains expressing GFP. Quantitative measure of intestinal fluorescence intensity at day 4 post infection is represented as the mean and standard error of the means from at least 20 worms. Data are normalized to wild type levels. (B) Representative nematode intestinal colonization micrograph of J96 and its *hfq* mutant at day 4. (C) Growth phenotypes of wild type and *hfq* mutant strains in liquid NGM media. (D) Influence of *hfq* deletion on sensitivity towards oxidative stress.

relevant in the *C. elegans* model, are regulated by Hfq. The fact that *hfq* mutants of all pathotypes of *E. coli* do grow and colonize the nematode intestine, yet being significantly reduced in pathogenicity, allows us to hypothesize that interfering with the Hfq regulon would be an interesting concept in development of antivirulence-like compounds against *E. coli*. Knowing that Hfq is highly conserved in *E. coli* and in several species in the phylum, including commensals, interfering with Hfq function may have detrimental rather than beneficial effects on the gut microbiome. However, infections at extraintestinal sites, such as the urinary tract, may be relieved by factors acting on Hfq. It has been shown that the *C. elegans* infection model can be used in the assessment of treatment efficacy on uropathogenic *E. coli* by phytochemicals established for human consumption [25]. In this respect, the nematode could also be used as a simple in vivo model to study the potency of factors targeting Hfq or its regulon.

In conclusion, we have shown here that Hfq is required for *E. coli* pathogenesis in *C. elegans* irrespective of bacterial pathotype. Also, we find that lack of Hfq correlates with generally lowered nematode colonization ability and adaptation to environmental conditions. We observe, however, quite a difference in attenuation of bacterial pathogenicity in *C. elegans* between pathotypes with the UPEC strain being the most attenuated, suggesting that *E. coli* pathotypes or strains suffer differently in in vivo fitness or virulence expression due to loss of Hfq. Hfq regulatory function may serve as a future target of intervention against certain *E. coli* infections, and the *C. elegans* model may be useful for evaluation of treatment regimes.

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References

[1] J.B. Kaper, J.P. Nataro, H.L. Mobley, Pathogenic *Escherichia coli*, Nat. Rev. Microbiol. 2 (2004) 123–140.
[2] S. Escaich, Novel agents to inhibit microbial virulence and pathogenicity, Expert Opin. Ther. Pat. 20 (2010) 1401–1418.
[3] D.T. Hung, E.A. Shakhnovich, E. Pierson, J.J. Mekalanos, Small-molecule inhibitor of *Vibrio cholerae* virulence and intestinal colonization, Science 310 (2005) 670–674.
[4] E. Mylonakis, F.M. Ausubel, R.J. Tang, S.B. Calderwood, The art of serendipity: killing of *Caenorhabditis elegans* by human pathogens as a model of bacterial and fungal pathogenesis, Expert Rev. Anti. Infect. Ther. 1 (2003) 167–173.
[5] B. Squiban, C.L. Kurz, *C. elegans*: an all in one model for antimicrobial drug discovery, Curr. Drug Targets 12 (2011) 967–977.
[6] Y. Chao, J. Vogel, The role of Hfq in bacterial pathogens, Curr. Opin. Microbiol. 13 (2010) 24–33.
[7] Y. Liu, N. Wu, J. Dong, Y. Gao, X. Zhang, C. Mu, N. Shao, G. Yang, Hfq is a global regulator that controls the pathogenicity of *Staphylococcus aureus*, PLoS One 5 (2010) e13069.
[8] M.K. Chiang, M.C. Lu, L.C. Liu, C.T. Lin, Y.C. Lai, Impact of Hfq on global gene expression and virulence in *Klebsiella pneumoniae*, PLoS One 6 (2011) e22248.
[9] J.K. Christiansen, M.H. Larsen, H. Ingmer, L. Søgaard-Andersen, B.H. Kallipolitis, The RNA-binding protein Hfq of *Listeria monocytogenes*: role in stress tolerance and virulence, J. Bacteriol. 186 (2004) 3355–3362.
[10] S.A. Sousa, C.G. Ramos, L.M. Moreira, J.H. Leitão, The *hfq* gene is required for stress resistance and full virulence of *Burkholderia cepacia* to the nematode *Caenorhabditis elegans*, Microbiology 156 (2010) 896–908.
[11] N.M. Wilf, N.R. Williamson, J.P. Ramsay, S. Poulter, K.J. Bandyra, G.P. Salmond, The RNA chaperone, Hfq, controls two luxR-type regulators and plays a key role in pathogenesis and production of antibiotics in *Serratia* sp. ATCC 39006, Environ. Microbiol. 13 (2011) 2649–2666.
[12] J. Yamada, S. Yamasaki, K. Hirakawa, M. Hayashi-Nishino, A. Yamaguchi, K. Nishino, Impact of the RNA chaperone Hfq on multidrug resistance in *Escherichia coli*, J. Antimicrob. Chemother. 65 (2010) 853–858.
[13] R.R. Kulesus, K. Diaz-Perez, E.S. Slechta, D.S. Eto, M.A. Mulvey, Impact of the RNA chaperone Hfq on the fitness and virulence potential of uropathogenic *Escherichia coli*, Infect. Immun. 76 (2008) 3019–3026.
[14] M.M. Kendall, C.C. Gruber, D.A. Rasko, D.T. Hughes, V. Sperandio, Hfq virulence regulation in enterohemorrhagic *Escherichia coli* O157:H7 strain 86-24, J. Bacteriol. 193 (2011) 6843–6851.
[15] E.A. Shakhnovich, B.M. Davis, M.K. Waldor, Hfq negatively regulates type III secretion in EHEC and several other pathogens, Mol. Microbiol. 74 (2007) 347–363.
[16] K.T. Simonsen, G. Nielsen, J.V. Bjerrum, T. Kruse, B.H. Kallipolitis, J. Møller-Jensen, A role for the RNA chaperone Hfq in controlling adherent-invasive *Escherichia coli* colonization and virulence, PLoS One 6 (2011) e16387.
[17] T.I. Moy, A.R. Ball, Z. Anklesaria, G. Casadei, K. Lewis, F.M. Ausubel, Identification of novel antimicrobials using a live-animal infection model, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 10414–10419.
[18] T. Stiernagle, Maintenance of *C. elegans*, in: The *C. elegans* Research Community, WormBook, February 11, 2006. http://dx.doi.org/10.1895/wormbook.1.101.1, http://www.wormbook.org.
[19] B.P. Cormack, R.H. Valdivia, S. Falkow, FACS-optimized mutants of the green fluorescent protein (GFP), Gene 173 (1996) 33–38.
[20] M.K. Chaveroche, J.M. Ghigo, C. d'Enfert, A rapid method for efficient gene replacement in the filamentous fungus *Aspergillus nidulans*, Nucleic Acids Res. 28 (2000) E97.
[21] M. Diard, S. Baeriswyl, O. Clermont, S. Gouriou, B. Picard, F. Taddei, E. Denamur, I. Matic, *Caenorhabditis elegans* as a simple model to study phenotypic and genetic virulence determinants of extraintestinal pathogenic *Escherichia coli*, Microbes Infect. 9 (2007) 214–223.
[22] Y. Lee, Y. Kim, S. Yeom, S. Kim, S. Park, C.O. Jeon, W. Park, The role of disulfide bond isomerase A (DsbA) of *Escherichia coli* O157:H7 in biofilm formation and virulence, FEMS Microbiol. Lett. 278 (2008) 213–222.
[23] J. Hwang, L.M. Mattei, L.G. VanArendonk, P.M. Meneely, I.N. Okeke, A pathoadaptive deletion in an enteroaggregative *Escherichia coli* outbreak strain enhances virulence in a *Caenorhabditis elegans* model, Infect. Immun. 78 (2010) 4068–4076.
[24] H.C. Tsui, H.C. Leung, M.E. Winkler, Characterization of broadly pleiotropic phenotypes caused by an *hfq* insertion mutation in *Escherichia coli* K-12, Mol. Microbiol. 13 (1994) 35–49.
[25] J.P. Lavigne, G. Bourg, C. Combescure, H. Botto, A. Sotto, In-vitro and in-vivo evidence of dose-dependent decrease of uropathogenic *Escherichia coli* virulence after consumption of commercial *Vaccinium macrocarpon* (cranberry) capsules, Clin. Microbiol. Infect. 14 (2008) 350–355.

Manuscript 2

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The Alkaloid Compound Harmane Increases the Lifespan of *Caenorhabditis elegans* during Bacterial Infection, by Modulating the Nematode's Innate Immune Response

Henrik Jakobsen[1,2☯], Martin S. Bojer[1,2☯], Martin G. Marinus[3], Tao Xu[3], Carsten Struve[2], Karen A. Krogfelt[2], Anders Løbner-Olesen[4]*

1 Department of Science, Systems and Models, Roskilde University, Roskilde, Denmark, **2** Department of Microbiology and Infection Control, Statens Serum Institut, Copenhagen, Denmark, **3** Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, Massachusetts, United States of America, **4** Department of Biology, University of Copenhagen, Copenhagen, Denmark

Abstract

The nematode *Caenorhabditis elegans* has in recent years been proven to be a powerful in vivo model for testing antimicrobial compounds. We report here that the alkaloid compound Harmane (2-methyl-β-carboline) increases the lifespan of nematodes infected with a human pathogen, the Shiga toxin-producing *Escherichia coli* O157:H7 strain EDL933 and several other bacterial pathogens. This was shown to be unrelated to the weak antibiotic effect of Harmane. Using GFP-expressing *E. coli* EDL933, we showed that Harmane does not lower the colonization burden in the nematodes. We also found that the expression of the putative immune effector gene *F35E12.5* was up-regulated in response to Harmane treatment. This indicates that Harmane stimulates the innate immune response of the nematode; thereby increasing its lifespan during bacterial infection. Expression of *F35E12.5* is predominantly regulated through the p38 MAPK pathway; however, intriguingly the lifespan extension resulting from Harmane was higher in p38 MAPK-deficient nematodes. This indicates that Harmane has a complex effect on the innate immune system of *C. elegans*. Harmane could therefore be a useful tool in the further research into *C. elegans* immunity. Since the innate immunity of *C. elegans* has a high degree of evolutionary conservation, drugs such as Harmane could also be possible alternatives to classic antibiotics. The *C. elegans* model could prove to be useful for selection and development of such drugs.

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* E-mail: lobner@bio.ku.dk

☯ These authors contributed equally to this work.

Introduction

For more than a decade now, the nematode *Caenorhabditis elegans* has been used as a simple infection model for several important human pathogens [1]. In recent years the nematode has also been used as an in vivo model to screen for compounds that combat these microbial infections [2,3]. The nematode constitutes an attractive model since it allows identification of classic antibiotic compounds, as well as compounds that inhibit bacterial virulence or stimulate the nematode's immune response. An example of such a compound, targeting the innate immune system of *C. elegans*, has recently been reported by Pukkila-Worley *et al.* [4]. They showed that the small molecule drug, named RPW-24, up-regulated several antimicrobial immune effector genes; resulting in increased lifespan of the worms, when infected with the pathogen *Pseudomonas aeruginosa*.

There are at least four pathways regulating immunity of *C. elegans*. These are the transforming growth factor-ß-like pathway, the p38 mitogen-activated protein kinase pathway (p38 MAPK pathway), the insulin-like receptor pathway, and the programmed cell death pathway [5]. Two of these are of particular interest, since they are known to promote longevity in *C. elegans* during infections of the intestine: the p38 MAPK pathway and the insulin-like receptor pathway. The p38 MAPK pathway has been shown to be induced in response to infection by specific human pathogens such as *P. aeruginosa* [6] and *Yersinia pestis* [7]. In contrast, the insulin-like receptor pathway is believed to provide the nematode with a continuous low-level protection against a broad range of pathogens [6,8]. Apart from antimicrobial factors, these pathways also control the expression of proteins involved in protection against environmental stress.

Here we report that the alkaloid compound Harmane (2-methyl-β-carboline) increases the lifespan of *C. elegans* during infection by several pathogenic bacteria. Harmane was initially found as a hit in a compound library screen, using a bacterial two hybrid assay. In the screen we were trying to identify inhibitors of the interaction between the enterohemorrhagic *E. coli* virulence factors, Tir (translocated intimin receptor) and Intimin [9] (Method S1). We wanted to use the nematode model to verify

the importance of the virulence factor and to show any in vivo efficacy of Harmane. We found that the virulence factor did not contribute to the pathogenicity of the bacteria against the nematode. However, we observed a marked increase in the lifespan of nematodes feeding on bacteria grown in the presence of Harmane (Figure S1). This led us to suspect that Harmane targeted an unknown virulence factor in the bacteria. Further studies, however, revealed that the target of Harmane was not in the bacteria, but in *C. elegans*. Based on our results we suggest that Harmane stimulates the immune/stress response in the nematode.

Results and Discussion

Harmane promotes longevity in C. elegans, infected with E. coli EDL933, in a dose dependent manner - unrelated to its antibiotic effect

After discovering that Harmane had the ability to promote survival of C. elegans, infected with E. coli EDL933; we decided to investigate this effect more closely. We tested the effect of Harmane concentration on survival. This was performed in a standard agar-based infection assay, with an immuno-compromised mutant of C. elegans. This strain carries a mutation in the sek-1 gene of the p38 MAPK pathway, making it more susceptible to several pathogens [3,10]. We found that the median survival of the nematodes changed from 7 days on the solvent DMSO or 5 µM Harmane, to 9 days on 50 µM Harmane and 13 days on 150 µM Harmane (Figure 1A). The lifespan of C. elegans did not change significantly at higher concentrations of Harmane (see Figure S1).We speculated whether this effect could be explained by a mere antimicrobial effect of Harmane. Harmane has previously been reported to have a minor antimicrobial effect against E. coli [11]. Reza et al. reported a minimum inhibitory concentration (MIC) of 0.6 mg/ml (3.29 mM). We found the MIC to be 0.5 mg/ml (2.74 mM) in nematode growth medium (NGM) (see Table S1 and Method S2). This was much higher than the highest concentration used in our assays, however, we decided to compare the antimicrobial effect of Harmane, to a comparable concentration of the traditional antibiotic tetracycline, in the survival assay. We grew E. coli EDL933 in triplicate in NGM medium, with different concentrations of Harmane or a low concentration of tetracycline, and plotted the number of colony forming units (CFU) against time (Figure 1B). Both Harmane and tetracycline had an inhibitory effect, on the growth of the bacteria. However, the inhibitory effect of 0.1 µg/ml tetracycline was stronger than that of both 50 and 150 µM of Harmane. We decided to compare the lifespan of C. elegans on plates with 0.1 µg/ml tetracycline and 150 µM of Harmane. We found that tetracycline had only a minor effect on the lifespan of the nematode, compared to Harmane (Figure 1C). This is similar to an earlier report, which found that tetracycline only rescued C. elegans from the pathogen Enterococcus faecalis, at concentrations several fold higher than the MIC [3]. We concluded that the lifespan extension from Harmane was not caused by its antimicrobial effect.

Harmane extends the lifespan of sek-1 worms on several pathogenic bacteria as well as heat-killed E. coli EDL933

At this point we still believed that the effect of Harmane was exerted on the bacteria, possibly by targeting an unknown virulence factor. In order to confirm this suspicion, we decided to test the ability of Harmane to rescue nematodes infected with other bacterial pathogens. We reasoned that if the target of Harmane was in the bacteria, it would be possible to find strains that lacked this target. We chose three pathogens known to be lethal to C. elegans: Salmonella serovar Typhimurium strain C17

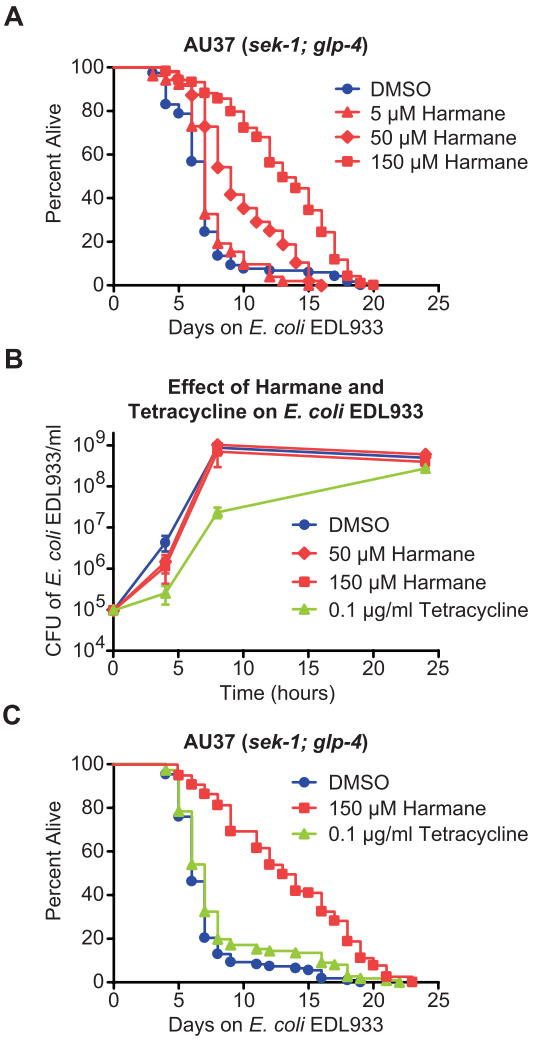

Figure 1. The effect of Harmane is dose-dependent, but unrelated to its antimicrobial effect. (A) *E. coli* EDL933 infection assay on *sek-1*; *glp-4* nematodes with different concentrations of Harmane compared to the solvent (DMSO). The lifespan extension is significant for the two highest concentrations of Harmane compared to DMSO ($P<0.0001$) [DMSO, n = 118; 5 µM, n = 52; 50 µM, n = 48; 150 µM, n = 119]. (B) Growth assay on *E. coli* EDL933 in NGM medium with different concentrations of Harmane or the solvent (DMSO), compared to *E. coli* EDL933 grown in NGM medium with 0.1 µg/ml of the antibiotic tetracycline. Both concentrations of Harmane affect the growth of EDL933, but not as much as 0.1 µg/ml of tetracycline. Data points are the average of three replicates and error bars represent SEM. (C) *E. coli* EDL933 infection assay on *sek-1*; *glp-4* nematodes with comparable concentrations of Harmane and tetracycline, compared to DMSO. Tetracycline, at 0.1 µg/ml, extends the lifespan significantly ($P = 0.0283$) as well does 150 µM Harmane ($P<0.0001$). However, the mean survival of nematodes on DMSO is 6 days, compared to 7 days for 0.1 µg/ml tetracycline and 13 days for 150 µM Harmane [DMSO, n = 108; 0.1 µg/ml Tetracycline, n = 111; 150 µM Harmane n = 117].
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[12,13], Pseudomonas aeruginosa strain PA14 [14], and Enterococcus faecalis strain OG1RF [3,15]. We also reasoned that if the target was indeed a virulence factor, we would see no life extending effect of Harmane, if nematodes were fed on dead bacteria. However, we found that Harmane was able to rescue the nematodes on all three pathogenic strains and to promote longevity even on heat-killed E. coli EDL933 (Figure 2). Taken

together, these data indicated that the target of Harmane was in C. elegans, rather than in the pathogens. We had not noticed any avoidance behavior of the worms on plates with Harmane compared to the control plates with DMSO. However, we considered the possibility that Harmane could be toxic to the nematodes and alter their feeding behavior. We added Harmane or DMSO to the centre of lawns with non-pathogenic E. coli OP50. AU37 (sek-1; glp-4) nematodes were added to the lawns, and after 16 hours we scored the worms as either on the lawns or off the lawns (Figure S2 and Method S3). There was no significant difference between plates with Harmane and control plates (P = 0.2046). We concluded that Harmane was not toxic to C. elegans.

Harmane does not reduce the colonization-burden of E. coli EDL933 in the nematode intestine

We proceeded to determine whether Harmane caused a reduction in the colonization of the intestine of the nematode. A strong inverse correlation between the degree of bacterial colonization and the expected lifespan of the individual worms has recently been shown [16]. We expected that nematodes treated with Harmane would have an overall lower colonization-burden, compared to nematodes treated with DMSO. We used E. coli EDL933 carrying a plasmid expressing green fluorescent protein (GFP) [17], allowing us to visualize and quantify the bacteria inside the intestine (Figure 3A and 3B). Contrary to what we had expected, we found no difference in the colonization-burden, in worms treated with DMSO, and worms treated with Harmane. This prompted us to look for the Harmane target in the innate immune system of the nematode. Since all infection assays had been performed in the mutant nematode, with a defective p38 MAPK pathway; we reasoned that we should direct our attention towards the insulin-like receptor pathway.

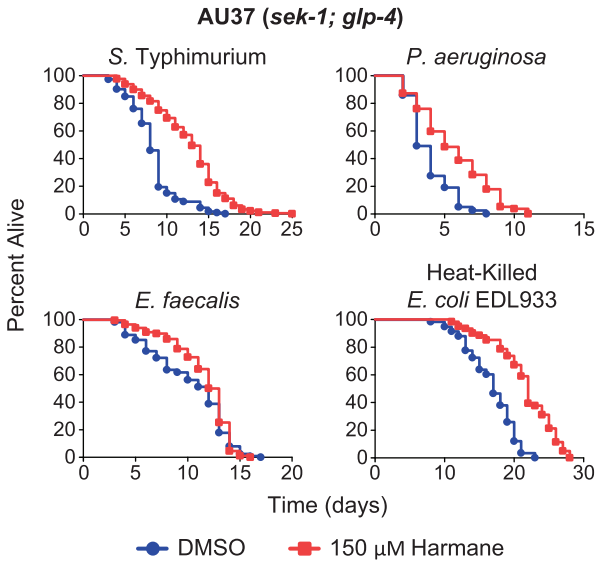

Figure 2. Harmane rescues *sek-1; glp-4* nematodes fed on different pathogens. There was a significant extension of life span of *C. elegans* feeding on bacteria grown on Harmane plates compared to plates with DMSO. The median survival when fed on *S.* Typhimurium was 8 days on DMSO and 13 days on 150 µM Harmane [$P<0.0001$; DMSO, n = 113; 150 µM Harmane, n = 180]. When fed on *P. aeruginosa* on DMSO, median survival was 3 days, and on 150 µM Harmane it was 5 days [$P<0.0001$; DMSO; n = 120; 150 µM Harmane, n = 134]. Feeding on *E. faecalis* on DMSO resulted in a median survival of 12 days, compared to 12.5 days on 150 µM Harmane [$P = 0.0013$; DMSO, n = 162; 150 µM Harmane, n = 198]. When feeding on heat-killed *E. coli* EDL933, the median survival was 17 days on DMSO, compared to 22 days on 150 µM Harmane [$P<0.0001$; DMSO, n = 58; 150 µM Harmane, n = 61].
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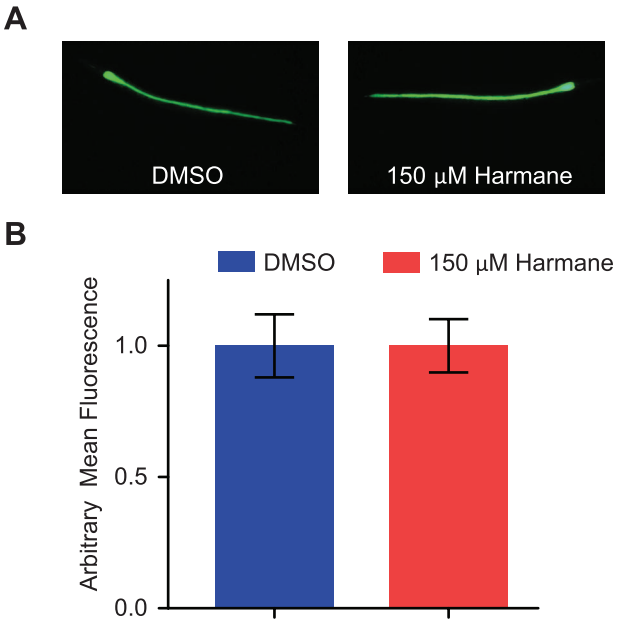

Figure 3. Harmane does not reduce the colonization-burden in *C. elegans* by *E. coli* EDL933. (A) Fluorescence microscopy pictures of *sek-1; glp-4* mutant *C. elegans* after feeding 4 days on GFP-expressing *E. coli* EDL933, grown on either DMSO or Harmane. The pictures show examples of strongly colonized individuals. (B) Individual nematodes in fluorescence pictures were quantified, and data normalized to the level of the DMSO treated nematodes. There was no significant difference between the two samples [DMSO, n = 56; 150 µM Harmane, n = 69; error bars indicate SEM].
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Harmane does not target the insulin-like receptor pathway of C. elegans

The insulin-like receptor pathway regulates the entry of C. elegans into the very long-lived dauer larval stage, instead of the normal L3 larval stage. The decision to enter the dauer stage must normally be made in the L1 larval stage. However, it has been shown that mutations in the daf-2 gene of the pathway also can cause fertile, active, adult nematodes to more than double their lifespan [18]. It is therefore believed that the insulin-like receptor pathway is responsible for regulating the nematodes basic response to environmental stress [8,19]. This stress can be in the form of low food resources or pathogenic bacteria. Under such unfavorable conditions, the insulin-like receptor pathway is activated; as a result resources are diverged away from growth and reproduction, and towards stress resistance and longevity. The insulin-like receptor pathway controls the transcription of genes involved in stress resistance via the transcription factor DAF-16. The lifespan extending effect of daf-2 mutants has been shown to be completely suppressed by mutations in daf-16 [18]. We reasoned therefore, that if Harmane acted on the insulin-like receptor pathway, Harmane would have no effect on a daf-16; glp-1 mutant nematode. We tested the susceptibility of this strain to E. coli EDL933, with DMSO or 150 µM Harmane. We found that the effect of Harmane on the lifespan of daf-16; glp-1 mutants was considerably less pronounced, than in the sek-1; glp-4 mutant,

although exposure to Harmane still resulted in significantly longer lifespan (Figure 4A). This indicated a possible interaction between the insulin-like receptor pathway and Harmane. In order to investigate this possible involvement of the insulin-like receptor pathway further, we assumed that activation of this pathway would also negatively affect traits such as, pharyngeal activity and body length of C. elegans. We performed the measurements of pharyngeal activity and body length on the sek-1; glp-4 mutant used in the first infection assay. We found no difference in the pharyngeal activity between nematodes exposed to Harmane compared to DMSO (Figure 4B). There was a small, but significant, difference in body length between nematodes exposed to Harmane for 1 day, compared to DMSO. However, such a difference could not be detected on day 4 (Figure 4C).

As mentioned above, activation of the insulin-like receptor pathway involves the transcription factor DAF-16. When not activated, DAF-16 is evenly distributed in the cytoplasm of all cells in the nematode. Upon activation DAF-16 is translocated from the cytoplasm to the nucleus. This translocation can be visualized using daf-16::gfp transgenic nematodes [19]. Transgenic nematodes were exposed to DMSO or Harmane for 1 hour or 20 hour, and then examined by fluorescence microscopy. As a positive control of DAF-16 translocation, we exposed the nematodes to 37°C for 30 minutes, just prior to examination. We detected no signs of DAF-16 translocation in response to Harmane (Figure 4D–E).

These results were contradictory to our first observation, which showed that the daf-16; glp-1 mutant was less affected by Harmane. Hence, we decided to test the effect of Harmane on a glp-4 nematode. We reasoned that if the results were similar to the ones found with the sek-1; glp-4 mutant, we could confirm the insulin-like receptor pathway as a target for Harmane. We tested the effect of Harmane on a SS104 C. elegans strain (this carries the glp-4 mutation conferring temperature sensitive sterility, but has intact immune pathways) in our survival assay. Contrary to the pronounced effect observed in sek-1; glp-4 nematodes, we found that the response of glp-4 nematodes to Harmane mirrored the minor response of the daf-16; glp-1 nematodes (Figure 5). There was only a one day extension of lifespan, as a result of Harmane. From this result we concluded that Harmane does not affect the insulin-like receptor pathway.

Harmane induces the immune response gene F35E12.5

We wondered how it could be that Harmane had a much more pronounced effect in the sek-1; glp-4 mutant, than in the glp-4 nematode or the daf-16; glp-1 mutant. One explanation could be that Harmane activates the p38 MAPK pathway downstream of the SEK-1 protein. We did not consider this as a possibility, since this effect would probably also be seen in the glp-4 worms and the daf-16; glp-1 mutant. Also, it was recently reported by Pukkila-Worley et al. that activation of the p38 MAPK pathway, by the small molecule RPW-24, resulted in a marked reduction in

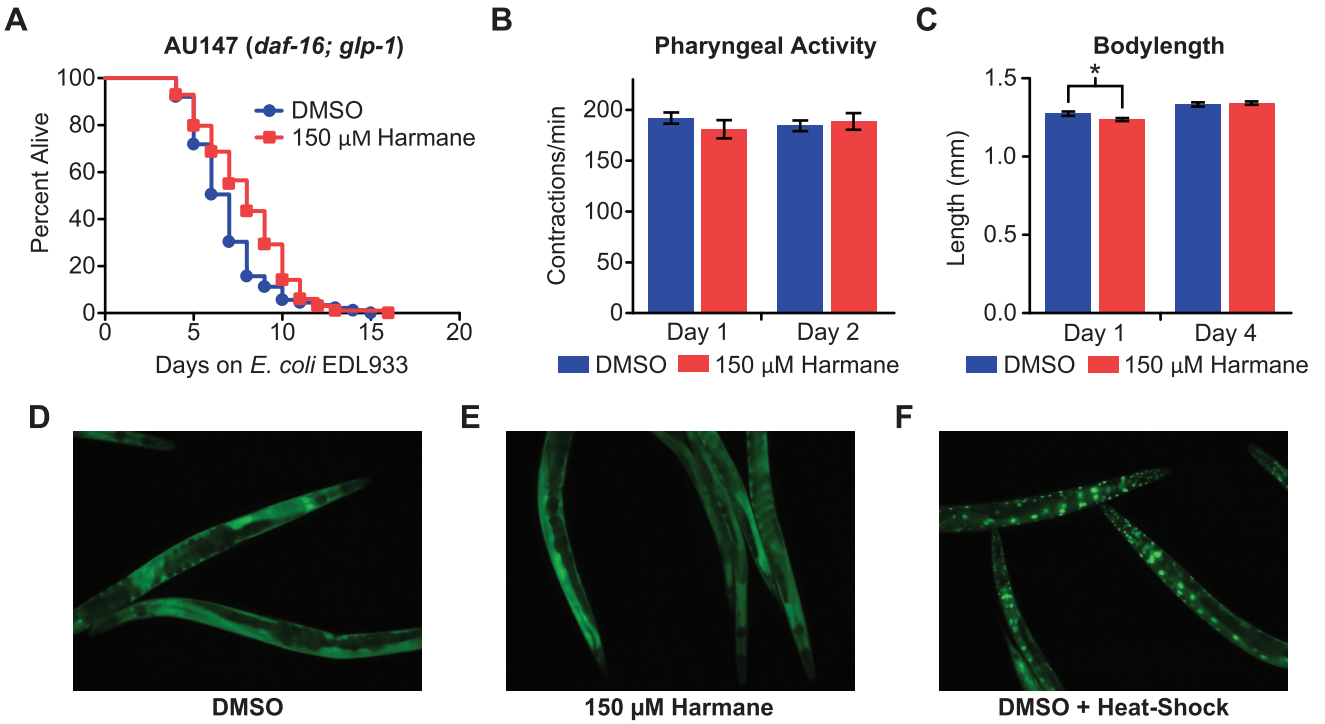

Figure 4. The effect of Harmane on the insulin-like receptor pathway. (A) *E. coli* EDL933 infection assay on *daf-16; glp-1* nematodes exposed to Harmane and DMSO. The median survival on Harmane was 8 days, compared to 7 days on DMSO. This was lower than the lifespan extension seen in *sek-1; glp-4* nematodes, however, still significantly different ($P<0.0001$) [DMSO, n = 89; 150 µM Harmane, n = 99]. (B) Pharyngeal pumping activity of *sek-1; glp-4* nematodes (AU37) fed on *E. coli* OP50, exposed to DMSO or Harmane [DMSO, n = 10; 150 µM Harmane, n = 10; error bars indicate SEM]. (C) Body length of *sek-1; glp-4* nematodes (AU37) fed on *E. coli* OP50, grown on DMSO or Harmane. Day 1 data shows a significant difference between Harmane and DMSO ($P = 0.0242$) [DMSO, day 1, n = 68; 150 µM Harmane, day 1, n = 72; DMSO, day 4, n = 72; 150 µM Harmane, day 4, n = 75; error bars indicate SEM]. (D) Fluorescence microscopy pictures of *daf-16::gfp* transgenic *C. elegans* after feeding 20 hours on *E. coli* OP50, grown on either DMSO or (E) 150 µSM Harmane. (F) Nematodes from a DMSO plate exposed to 37°C for 30 minutes, just prior to being analyzed (positive control of DAF-16 translocation to nucleus). We could not detect any effect of Harmane on DAF-16 in the worms, as a result of the Harmane treatment. Pictures taken after 1 hour exposure to DMSO or Harmane (not shown), were identical to the 20-hour pictures.
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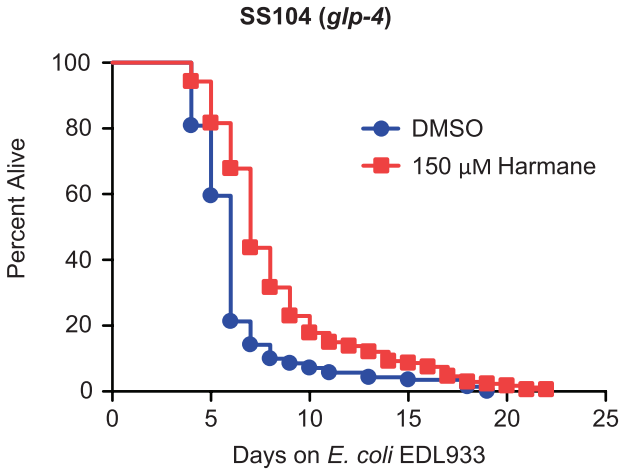

Figure 5. The effect of Harmane in *glp-4* nematodes. *E. coli* EDL933 infection assay with *glp-4* nematodes, exposed to DMSO or Harmane. The lifespan extension was from 6 days on DMSO to 7 days on Harmane [$P<0.0001$; DMSO, n = 141; 150 μM Harmane, n = 173].
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intestinal colonization by P. aeruginosa [4]. We did not observe such a decrease, in colonization with E. coli EDL933, with Harmane (Figure 3).

However, we decided to test the effect of Harmane on F35E12.5. This protein is a putative immune effector, and it has been found to be strongly induced during infection with P. aeruginosa [6] and Y. pestis, but not when fed on E. coli OP50 [7]. Troemel et al. and Bolz et al. found that this strong inducible expression of F35E12.5 was mediated by the p38 MAPK immune pathway. However, they also found weak inducible expression of F35E12.5 in pmk-1 mutant nematodes (these are defective in the p38 MAPK pathway, similar to sek-1 mutants). Again this induction was only seen on P. aeruginosa and Y. pestis, not on E. coli OP50. They proposed the existence of an immune pathway parallel to the p38 MAPK pathway.

To determine whether Harmane could induce F35E12.5, we exposed a wild-type C. elegans strain carrying a F35E12.5::gfp transgene [7] to Harmane or DMSO, for 20 hours. We analyzed the worms by fluorescence microscopy, and quantified the expression of F35E12.5::gfp (Figure 6A and 6B). We found a significantly stronger expression in the transgenic nematodes treated with Harmane ($P<0.0001$). This result was confirmed by qRT-PCR analysis on wild-type Bristol N2 nematodes, after 20 hours treatment with Harmane or DMSO. F35E12.5 was significantly up-regulated in response to Harmane ($P = 0.0175$). Thus, Harmane exhibits immune-inductive activity. However the up-regulation of F35E12.5 by Harmane in AY101 (F35E12.5::gfp) and Bristol N2 nematodes were many fold less than the up-regulation observed by Troemel et al. and Bolz et al. in response to P.aeruginosa and Y. pestis. This indicates that the p38 MAPK pathway is not the main target of Harmane.

Instead we propose the hypothesis that Harmane stimulates more than one pathway involved in pathogen resistance and longevity. This stimulon may include the p38 MAPK pathway. However, based on our results showing that Harmane has a much more pronounced effect in the sek-1; glp-4 mutant, than in the glp-4 or daf-16; glp-1 mutants, we hypothesize that Harmane targets one or more alternative immune responses and that these are being up-regulated in the absence of the p38 MAPK pathway. This phenomenon of up-regulation of immune effector genes in

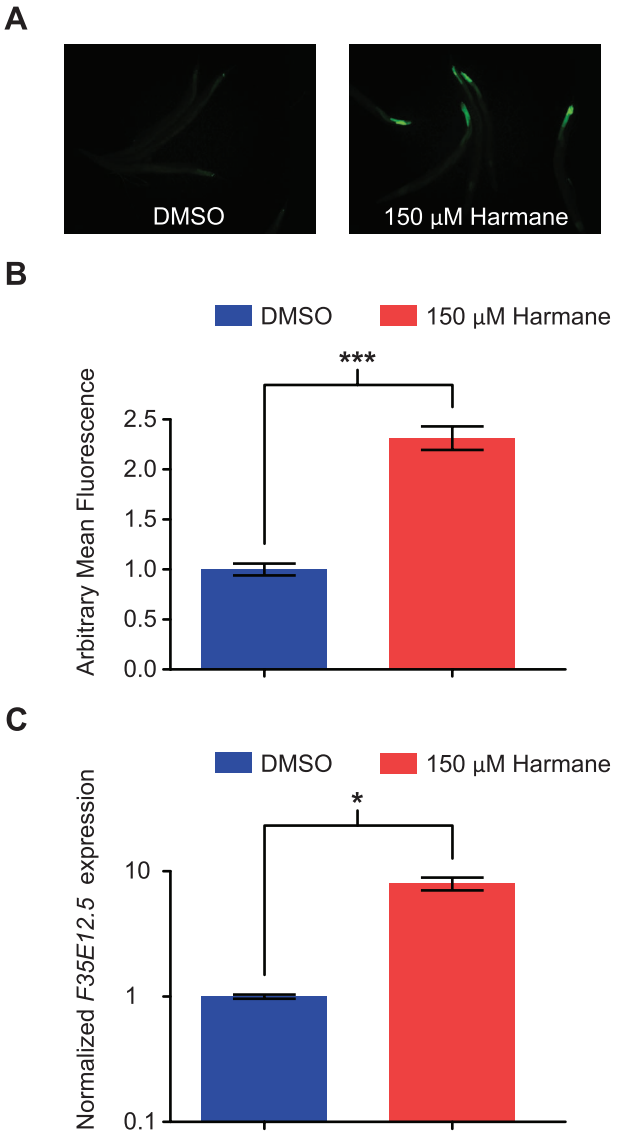

Figure 6. Harmane induces the immune response gene *F35E12.5*. (A) Fluorescence microscopy pictures of *F35E12.5::gfp* transgenic *C. elegans*, exposed to DMSO or Harmane for 20 hours, at 15°C (feeding on *E. coli* OP50). The fluorescence was always seen at the tail end of the animals. (B) Individual nematodes in fluorescence pictures were quantified, and data normalized to the level of the DMSO treated nematodes. There was a significant difference between the two samples [$P<0.0001$; DMSO, n = 54; 150 μM Harmane, n = 54; error bars indicate SEM]. (C) Verification of *F35E12.5* induction by Harmane by qRT-PCR. Bar graphs represent relative expression levels normalized to the control treatment (DMSO). 20 hours treatment with Harmane resulted in a significant induction ($P = 0.0175$) of *F35E12.5*. Error bars indicate SEM derived from two independent biological replicates.
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order to compensate for loss of genes encoding similar effectors has recently been reported in C. elegans [20].

Conclusion

We have shown here that the alkaloid compound Harmane increases the lifespan of nematodes infected with a human pathogen, the Shiga toxin-producing *Escherichia coli* EDL933 and several other bacterial pathogens. This was shown to be unrelated

to the weak antibiotic effect of Harmane. We found that the effect of Harmane was more pronounced in worms deficient in the p38 MAPK pathway (*sek-1*; *glp-4* mutants). Worms deficient in the insulin-like receptor pathway (*daf-16*; *glp-1* mutants) experienced the same minor lifespan extension as *glp-4* worms. We then demonstrated that Harmane induces the immune effector gene *F35E12.5*. This leads us to believe that Harmane stimulates the innate immune response of the nematode. We hypothesize that at least part of this stimulon involves other constituents than p38 MAPK and insulin-like signaling, however, these constituents and their activation by Harmane remains to be elucidated. Interestingly, the activity of Harmane did not lower the overall colonization-burden of live bacteria, despite rescuing the nematodes. Also, Harmane treatment increased the lifespan of worms feeding on non-viable bacteria. We therefore hypothesize that the response induced by Harmane is likely involved in general stress management rather than a direct antimicrobial response against pathogens. We believe that Harmane could be used as a tool to further investigate the complexity of the innate immune system of *C. elegans*; together with other immune-stimulatory drugs [4].

It may seem strange that an organism like *C. elegans*, which has evolved alongside numerous microorganisms, would benefit from outside drug intervention, targeting host innate immunity. However, *C. elegans* is not likely to meet many human pathogens in its natural environment (in the soil). It is conceivable that many of these pathogens do not trigger the innate immune system of the nematode. This is similar to the situation in humans, where for example *E. coli* is usually a commensal inhabitant of the large intestine. Immunomodulating drugs have therefore been proposed as a possible alternative to classic antibiotics [21]. Since the innate immune system of *C. elegans* has a high degree of evolutionary conservation, it is conceivable that results from the worm model could be extended to higher organisms, including humans. *C. elegans* could therefore prove useful for selection and development of such compounds. The immuno-stimulatory effect observed for Harmane could provide a scaffold upon which further elaborations on this paradigm are possible.

Materials and Methods

C. elegans and bacterial strains used

The Caenorhabditis elegans strains used in this study were: C. elegans N2 Bristol [22], C. elegans AU37 [sek-1(km4); glp-4(bn2) I] (MAPK kinase deficiency and temperature-sensitive sterile) [3], C. elegans AU147 [daf-16(mgDf47) I; glp-1(e2141) III] (transcription factor DAF-16 deficiency and temperature-sensitive sterile), C. elegans SS104 [glp-4(bn2) I] (temperature-sensitive sterile), C. elegans TJ356 [zIs356 [daf-16::gfp+ rol-6(su1006)] [19] and C. elegans AY101 [F35E12.5p::gfp+rol-6(su1006)] [7]. All C. elegans strains were maintained and propagated on NGM media [23], with E. coli OP50 as food source.

The bacterial strains used were: Escherichia coli OP50, E. coli O157:H7 strain EDL933 [24] (Shiga toxin-producing E. coli strain), Salmonella Typhimurium strain C17 [12,13], Pseudomonas aeruginosa strain PA14 [14] and Enterococcus faecalis strain OG1RF [3,15]. Bacterial strain were grown in LB media at 37°C, except E. faecalis which was grown in brain hearth infusion media (BHI) (OXOID Ltd.) at 37°C.

C. elegans bacterial infection assays

A synchronous population of nematodes was obtained, by releasing worm embryos using alkaline hypochlorite treatment [23]; followed by hatching of the eggs and L1 arrest in M9 buffer at 15°C overnight. Synchronous L1 larvae were transferred to nematode growth medium (NGM) agar plates [23] seeded with OP50 and allowed to develop into sterile adult/L4 larvae, by incubating for two days at 25°C. Hereafter they were washed three times in M9 buffer and transferred to assay plates, with bacterial lawns. Assay plates were prepared on 60-mm culture plates with NGM agar supplemented with 0.3% DMSO or the indicated concentrations of Harmane (Sigma-Aldrich, CAS Number 486-84-0). Exceptions to this were the plates for Enterococcus faecalis, which were BHI agar (OXOID Ltd., supplemented with 5 mg/ml cholesterol) and the plates for heat-killed E. coli EDL933, which were NGM agar supplemented with 25 μg/ml of chloramphenicol (in order to inhibit any viable E. coli EDL933 or residual E. coli OP50).

The plates were seeded with 20 μl of overnight culture, of bacterial strains, followed by overnight incubation at 37°C. After acclimatization of the plates to room temperature, about 50 worms were transferred to each plate. Plates were incubated at 25°C and scored for dead worms each day. A worm was considered dead when it failed to respond to a touch with a platinum wire; dead worms stuck to the wall of the plate were censored from analysis. Survival data from duplicate or triplicate plates were pooled and subjected to survival analysis. Results presented are representative of repeated independent assays.

Growth assays with E. coli EDL933 subjected to Harmane or tetracycline

The growth kinetics of E. coli EDL933 in the presence of varying concentrations of Harmane or the antibiotic tetracycline was determined in NGM medium. An overnight culture of E. coli EDL933 was diluted into fresh NGM medium, supplemented with 0.3% DMSO or Harmane at the concentrations: 50 μM and 150 μM. Tetracycline was tested at a concentration of 0.1 μg/ml. All cultures were prepared in triplicate. The cultures were incubated at 37°C, with rigorous shaking and samples were taken at 4, 8 and 24 hours. Samples were serially diluted and 5 μl of each dilution spotted on an LB-agar plate. The plate was incubated overnight at 37°C.

Visualization and quantification of bacterial intestinal colonization

Synchronised adult/L4 stage C. elegans AU37 (sek-1; glp-4) were transferred from lawns of E. coli OP50 to lawns of E. coli EDL933 carrying pBAD18-GFP, on NGM plates containing 100 μg/ml ampicillin and 0.2% arabinose (in order to activate expression of GFP from the P_{BAD} promoter) and either 0.3% DMSO or 150 μM Harmane. On day 4 the worms were washed from the plates into M9 media followed by 3 further washes to remove external bacteria. Subsequently, the worms were anaesthetized and immobilized, by addition of 1% NaN_3, and placed on top of a 1.5% agarose pad on a microscope slide. Worms were examined and photographed with an Olympus BX61 microscope and an Olympus DP71 camera using the cellP software (Olympus). All photographs were acquired using the same settings and a fixed exposure time of 50 ms. Quantitative assessment of bacterial colonization was done by determination of fluorescence intensity of nematodes subjected to 0.3% DMSO or 150 μM Harmane, by analysis of individual nematodes, using ImageJ v1.45.

Analysis of pharyngeal pumping activity

About 30 synchronised L4/young adult C. elegans AU37 (sek-1; glp-4) nematodes were transferred to lawns of E. coli OP50, on NGM plates with either 0.3% DMSO or 150 μM Harmane. After 24 and 48 hours incubation at 25°C, the worm's pharyngeal

grinder activity was measured. We observed the grinder activity of a single adult for 20 sec, using an Olympus SZX7 stereomicroscope. We counted the number of contractions of the terminal bulb, of the grinder. This was done for 10 nematodes on each plate, each day.

Measuring body length of C. elegans

Synchronised adult/L4 stage C. elegans AU37 (sek-1; glp-4) were transferred to E. coli OP50 lawns, on NGM plates containing either 0.3% DMSO or 150 μM Harmane. On day 1 and 4 the worms were washed from plates into M9 media followed by a single wash to remove external bacteria. The worms were then anaesthetized and immobilized, by addition of 1% NaN_3, and placed on top of a 1.5% agarose pad on a microscope slide. Worms were examined and photographed with an Olympus SZX7 stereomicroscope and an Olympus SC30 camera, using the Analysis getIT software (Olympus). The length of the worms was measured using ImageJ v1.45, as described in [25]. The sample sizes are given in the legend to figure 4.

DAF-16 translocation assay

Synchronised adult/L4 stage C. elegans TJ356 (daf-16::gfp) were transferred to lawns of E. coli OP50 on plates with either 0.3% DMSO or 150 μM Harmane. The plates were incubated at 15°C for 1 hour or 20 hours. Then the worms were washed from the plates into M9 media followed by a wash to remove external bacteria. Subsequently, the worms were anaesthetized and immobilized, by addition of 1% NaN_3, and placed on top of a 1.5% agarose pad on a microscope slide. Worms were examined and photographed with an Olympus BX61 microscope and an Olympus DP71 camera using the celÎP software (Olympus). In each experiment (1-hour or 20-hour) we placed one of the DMSO plates at 37°C for 30 minutes, just prior to the nematodes being analysed. This served as a positive control of DAF-16 translocation to the nucleus. We compared the nematodes treated with Harmane to the positive control to look for signs of DAF-16 translocation.

F35E12.5-GFP visualization and quantification

Synchronised adult/L4 stage C. elegans AY101 (F35E12.5::gfp) were transferred to lawns of E. coli OP50 on plates with either 0.3% DMSO or 150 μM Harmane. The plates were incubated at 15°C for 20 hours. The worms were then washed from the plates into M9 media followed by a wash to remove external bacteria. The worms were anaesthetized and immobilized, by addition of 1% NaN_3, and placed on top of a 1.5% agarose pad on a microscope slide. Worms were examined and photographed with an Olympus BX61 microscope and an Olympus DP71 camera using the celÎP software (Olympus). All photographs were acquired using the same settings and a fixed exposure time. Quantitative assessment of GFP expression was done by determination of fluorescence intensity of nematodes subjected to DMSO or 150 μM Harmane, by analysis of individual nematodes, using ImageJ v1.45.

Quantitative RT-PCR

Synchronised Bristol N2 nematodes (100–200 animals) fed on OP50 on plates with Harmane or solvent (DMSO) for 20 hours were washed off the plates and transferred into RLT Plus Buffer (Qiagen). Sterile RNase-free bashing beads were added, followed by lysis and homogenization in a TissueLyser II (Qiagen) for 5 min. RNA extraction was hereafter performed with RNeasy Plus Mini Kit (Qiagen) combined with on-column DNase treatment (RNase-Free DNase Set, Qiagen). cDNA was synthesized by the SuperScript III First-Strand Synthesis SuperMix (Invitrogen). Relative expression levels of F35E12.5 was determined and normalized to pan-actin (act-1, -3, -4) using the QuantiTect SYBR Green PCR Kit (Qiagen), a Stratagene MX3000P qPCR machine, and previously published primers [7].

Statistics

Differences in the survival of C. elegans, in the infection assays, were determined using GraphPad Prism version 5.00 (www.graphpad.com). The Kaplan-Meier method was used to calculate survival fractions and log-rank test was used to compare survival curves. Mean fluorescence of worms feeding on GFP expressing E. coli EDL933 were compared by unpaired, two-tailed Students t-test, using GraphPad Prism. Data for 'pharyngeal pump activity'and 'body length' were also analysed by unpaired, two-tailed Students t-test in GraphPad Prism. Gene expression fold change from qRT-PCR analysis was analysed using unpaired, two-tailed Students t-test in GraphPad Prism. Sample sizes for the different assays are given in the figure legends. Values of $P \leq 0.05$ were considered statistically significant.

Supporting Information

Figure S1 The Intimin and Tir interaction only plays a minor role in pathogenicity of E. coli EDL933 towards C. elegans. Harmane strongly extends lifespan.
(PDF)

Figure S2 C. elegans AU37 nematodes show no avoidance behavior against Harmane.
(PDF)

Table S1 Minimum inhibitory concentrations of tetracycline and Harmane in NGM media towards E. coli EDL933, S. Typhimurium C17, P. aeruginosa PA14 and E. faecalis OG1RF.
(PDF)

Method S1 Two-hybrid screen for inhibitors of the Intimin and Tir (translocated intimin receptor) interaction.
(PDF)

Method S2 Determination of minimum inhibitory concentration.
(PDF)

Method S3 Avoidance assay for C. elegans.
(PDF)

Acknowledgments

We thank Kim F. Rewitz and Julie L. Hentze from University of Copenhagen for use of equipment and for assistance with the qRT-PCR experiments. We also thank Hong Cao and the University of Massachusetts Medical School Small Molecule Screening Core Facility for their help in the two-hybrid screen. The *C. elegans* strains used in this study were obtained from the Caenorhabditis Genetics Center (CGC), which is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440).

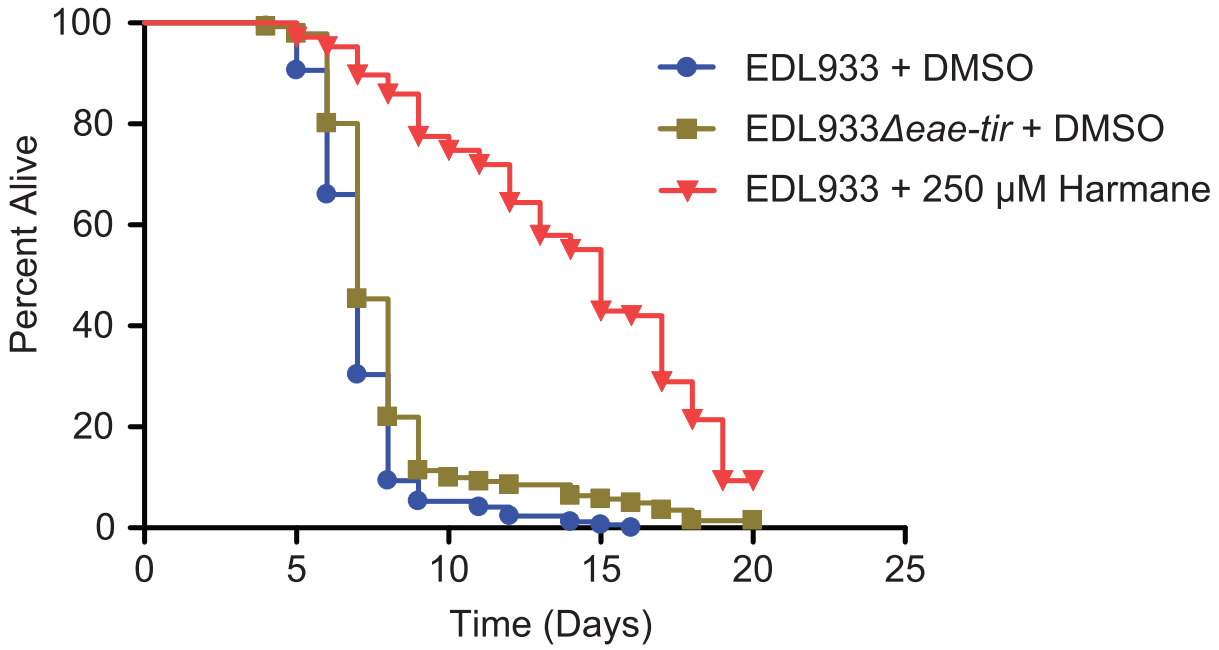
Author Contributions

Conceived and designed the experiments: HJ MSB MGM TX CS KAK ALO. Performed the experiments: HJ MSB MGM TX. Analyzed the data: HJ MSB MGM TX. Wrote the paper: HJ MSB MGM CS KAK ALO.

References

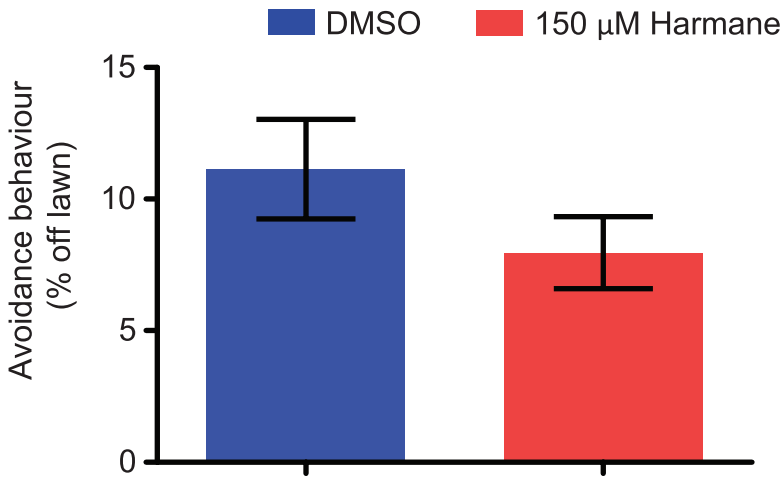
1. Sifri CD, Begun J, Ausubel FM (2005) The worm has turned--microbial virulence modeled in *Caenorhabditis elegans*. Trends Microbiol 13: 119-127.
2. Breger J, Fuchs BB, Aperis G, Moy TI, Ausubel FM, et al. (2007) Antifungal chemical compounds identified using a *C. elegans* pathogenicity assay. PLoS Pathog 3: e18.
3. Moy TI, Ball AR, Anklesaria Z, Casadei G, Lewis K, et al. (2006) Identification of novel antimicrobials using a live-animal infection model. Proc Natl Acad Sci U S A 103: 10414-10419.
4. Pukkila-Worley R, Feinbaum R, Kirienko NV, Larkins-Ford J, Conery AL, et al. (2012) Stimulation of Host Immune Defenses by a Small Molecule Protects *C. elegans* from Bacterial Infection. PLoS Genet 8: e1002733.
5. Schulenburg H, Kurz CL, Ewbank JJ (2004) Evolution of the innate immune system: the worm perspective. Immunol Rev 198: 36-58.
6. Troemel ER, Chu SW, Reinke V, Lee SS, Ausubel FM, et al. (2006) p38 MAPK regulates expression of immune response genes and contributes to longevity in *C. elegans*. PLoS Genet 2: e183.
7. Bolz DD, Tenor JL, Aballay A (2010) A conserved PMK-1/p38 MAPK is required in *Caenorhabditis elegans* tissue-specific immune response to *Yersinia pestis* infection. J Biol Chem 285: 10832-10840.
8. Shivers RP, Youngman MJ, Kim DH (2008) Transcriptional responses to pathogens in *Caenorhabditis elegans*. Curr Opin Microbiol 11: 251-256.
9. Campellone KG, Leong JM (2003) Tails of two Tirs: actin pedestal formation by enteropathogenic *E. coli* and enterohemorrhagic *E. coli* O157:H7. Curr Opin Microbiol 6: 82-90.
10. Kim DH, Feinbaum R, Alloing G, Emerson FE, Garsin DA, et al. (2002) A conserved p38 MAP kinase pathway in *Caenorhabditis elegans* innate immunity. Science 297: 623-626.
11. Reza VRM, Abbas H (2007) Cytotoxicity and Antimicrobial Activity of Harman Alkaloids. Pharmacology and Toxicology 2: 677-680.
12. Aballay A, Yorgey P, Ausubel FM (2000) *Salmonella typhimurium* proliferates and establishes a persistent infection in the intestine of *Caenorhabditis elegans*. Curr Biol 10: 1539-1542.
13. Labrousse A, Chauvet S, Couillault C, Kurz CL, Ewbank JJ (2000) *Caenorhabditis elegans* is a model host for *Salmonella typhimurium*. Curr Biol 10: 1543-1545.
14. Tan MW, Mahajan-Miklos S, Ausubel FM (1999) Killing of *Caenorhabditis elegans* by *Pseudomonas aeruginosa* used to model mammalian bacterial pathogenesis. Proc Natl Acad Sci U S A 96: 715-720.
15. Murray BE, Singh KV, Ross RP, Heath JD, Dunny GM, et al. (1993) Generation of restriction map of *Enterococcus faecalis* OG1 and investigation of growth requirements and regions encoding biosynthetic function. J Bacteriol 175: 5216-5223.
16. Portal-Celhay C, Bradley ER, Blaser MJ (2012) Control of intestinal bacterial proliferation in regulation of lifespan in *Caenorhabditis elegans*. BMC Microbiol 12: 49.
17. Bojer MS, Jakobsen H, Struve C, Krogfelt KA, Løbner-Olesen A (2012) Lack of the RNA chaperone Hfq attenuates pathogenicity of several *Escherichia coli* pathotypes towards *Caenorhabditis elegans*. Microbes Infect 14: 1034-1039.
18. Kenyon C, Chang J, Gensch E, Rudner A, Tabtiang R (1993) A *C. elegans* mutant that lives twice as long as wild type. Nature 366: 461-464.
19. Henderson ST, Johnson TE (2001) *daf-16* integrates developmental and environmental inputs to mediate aging in the nematode *Caenorhabditis elegans*. Curr Biol 11: 1975-1980.
20. Marsh EK, van den Berg MC, May RC (2011) A two-gene balance regulates *Salmonella* Typhimurium tolerance in the nematode *Caenorhabditis elegans*. PLoS One 6: e16839.
21. Hancock RE, Nijnik A, Philpott DJ (2012) Modulating immunity as a therapy for bacterial infections. Nat Rev Microbiol 10: 243-254.
22. Brenner S (1974) The genetics of *Caenorhabditis elegans*. Genetics 77: 71-94.
23. Stiernagle T (2006) Maintenance of *C. elegans*. WormBook: ed. The *C. elegans* Research Community, Wormbook, doi/10.1895/wormbook.1.101.1. Available: http://www.wormbook.org.
24. Riley LW, Remis RS, Helgerson SD, McGee HB, Wells JG, et al. (1983) Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. N Engl J Med 308: 681-685.
25. Morck C, Pilon M (2006) *C. elegans* feeding defective mutants have shorter body lengths and increased autophagy. BMC Dev Biol 6: 39.

Figure S1



The Intimin and Tir interaction only plays a minor role in pathogenicity of *E. coli* EDL933 towards *C. elegans*. Harmane strongly extends lifespan. Infection assay on *C. elegans* AU37 (*sek-1*; *glp-4*) nematodes with wild-type *E. coli* EDL933 grown on plates with 0.3% DMSO; *E. coli* EDL933Δ*eae-tir* (Lacking Intimin and Tir) grown on plates with 0.3% DMSO and *E. coli* EDL933 grown on plates with 250 µM Harmane. The curve for *E. coli* EDL933Δ*eae-tir* is significantly different ($P<0.001$) from the wild-type, but the median survival of nematodes was 7 days on both. The median survival of nematodes on 250 µM Harmane was 15 days. [Number of animals: *E. coli* EDL933 + DMSO, n=171; *E. coli* EDL933Δ*eae-tir* + DMSO, n=139; *E. coli* EDL933 + 250 µM Harmane, n=97].

Figure S2



***C. elegans* AU37 nematodes show no avoidance behavior against Harmane.** The average percentage of worms that were outside the bacterial lawn of *E. coli* OP50, supplemented with either DMSO or Harmane, after 16 hours of exposure. There was no significant difference between the two samples. [DMSO, n=365; 150 µM Harmane, n=644; error bars indicate SEM].

Table S1: Minimum inhibitory concentrations of tetracycline and Harmane in NGM media towards *E. coli* EDL933, *S.* Typhimurium C17, *P. aeruginosa* PA14 and *E. faecalis* OG1RF.

Strain	Growth	No Growth
E. coli EDL933	0.31 µg/ml Tetracycline	0.62 µg/ml Tetracycline
E. coli EDL933	0.25 mg/ml (1372 µM) Harmane	0.5 mg/ml (2744 µM) Harmane
S. Typhimurium C17	0.125 mg/ml (686 µM) Harmane	0.25 mg/ml (1372 µM) Harmane
P. aeruginosa PA14	0.5 mg/ml (2744 µM) Harmane	1 mg/ml (5488 µM) Harmane
E. faecalis OG1RF	0.5 mg/ml (2744 µM) Harmane	1 mg/ml (5488 µM) Harmane

Method S1: Two-hybrid screen for inhibitors of the Intimin and Tir (translocated intimin receptor) interaction.

Harmane was identified in a two-hybrid screen for inhibitors of the interaction between the enterohemorrhagic *E. coli* virulence factors, Tir and Intimin. The two-hybrid screen is based on the method devised by Karimova et al [1]where a stable interaction between the fusion subunits produces an active adenylate cyclase which activates transcription of the *lac* operon. We constructed plasmids pKT25-Tir and pUT18-Intimin using the full length coding sequence for each and strain BTH101 was transformed with these plasmids. The two vector plasmids, and pKT25-zip and pUT18-zip (containing a leucine zipper motif) in BTH101, served as the negative and positive controls respectively. The plasmid-containing strains were arrayed in 96 well plates in 200 µl of Davis and Mingioli minimal medium [2] supplemented with 0.2% maltose, ampicillin (50 µg/ml), kanamycin (25 µg/ml), thiamine (2 µg/ml), X-Gal (40 µg/ml) and IPTG (0.5 mM). The plates were incubated at 30°C and scanned at a wavelength of 664 nm after 48 and 72 hr on a Tecan Safire plate reader. Relative to the positive control, Harmane (10 µM) reduced the absorbance by 90% in BTH101 with the recombinant plasmids but had no effect on BTH101 with the leucine zipper control plasmids. Harmane for the screen was supplied by the University of Massachusetts Medical School Small Molecule Screening Core Facility which also performed the plate reading and data analysis.

1. Karimova G, Pidoux J, Ullmann A, Ladant D (1998) A bacterial two-hybrid system based on a reconstituted signal transduction pathway. Proc Natl Acad Sci U S A 95: 5752-5756.

2. Davis BD, Mingioli ES (1950) Mutants of *Escherichia coli* requiring methionine or vitamin B12. J Bacteriol 60: 17-28.

Method S2: Determination of minimum inhibitory concentration

The minimum inhibitory concentrations of Harmane and tetracycline were performed in analogy to standard broth microdilution procedures, with slight modifications. Test strains were inoculated (at approximately $5*10^5$ CFU/ml) into Nematode Growth Medium containing two-fold dilutions of Harmane or tetracycline followed by incubation at 37°C for 24 hours. *E. faecalis* was cultured under the same conditions, but in brain hearth infusion media (BHI) (OXOID). The lowest concentration preventing visual growth was noted.

Method S3: Avoidance assay for *C. elegans*.

C. elegans AU37 aversion behavior towards Harmane was evaluated essentially as previously described [1]. NGM plates seeded with a lawn of OP50 were instilled with 28 µl of 50 mM Harmane (final concentration 150 µM Harmane) or an equal volume of DMSO onto the center of the lawn and allowed to dry (1 hour at room-temperature). Hereafter, 50-100 synchronized adult animals were added to the center of the lawn and animals were scored as either on or off the lawn after 16 hours of incubation at 25°C. Data represent mean aversion behavior from six plates per condition and error bars correspond to SEM

1. Pukkila-Worley R, Feinbaum R, Kirienko NV, Larkins-Ford J, Conery AL, et al. (2012) Stimulation of Host Immune Defenses by a Small Molecule Protects *C. elegans* from Bacterial Infection. PLoS Genet 8: e1002733.