molecular microbiology

Laboratory adapted Escherichia coli K-12 becomes a pathogen of Caenorhabditis elegans upon restoration of O antigen biosynthesis.

Journal:	*Molecular Microbiology*
Manuscript ID:	MMI-2012-12368.R1
Manuscript Type:	Research Article
Date Submitted by the Author:	22-Nov-2012
Complete List of Authors:	Browning, Douglas; UB, Biosciences Wells, Timothy; University of Birmingham, Institute of Microbiology and Infection França, Fernanda; University of Birmingham, Institute of Microbiology and Infection Johnson, Matthew; University of Birmingham, Institute of Microbiology and Infection Morris, Faye; University of Birmingham, Institute of Microbiology and Infection Bryant, Jack; University of Birmingham, Institute of Microbiology and Infection Lund, Peter; University Of Birmingham, School of Biosciences Cunningham, Adam; only email imported, Hobman, Jon; University of Nottingham, School of Biosciences; May, Robin; School of Biosciences, University of Birmingham Edgbaston B15 2TT Webber, Mark; University of Birmingham, Immunity and Infection Henderson, Ian; University of Birmingham, Immunity and Infection
Key Words:	Escherichia coli K-12, lipopolysaccharide, O antigen, Caenorhabditis elegans

Laboratory adapted *Escherichia coli* K-12 becomes a pathogen of *Caenorhabditis elegans* upon restoration of O antigen biosynthesis.

Douglas F. Browning[1*†], Timothy J. Wells[1†], Fernanda L. S. França[1], Faye C. Morris[1], Yanina R. Sevastsyanovich[1], Jack A. Bryant[2], Matthew D. Johnson[2], Peter A. Lund[2], Adam F. Cunningham[1], Jon L. Hobman[3], Robin C. May[2], Mark A. Webber[1] and Ian R. Henderson[1*].

[1]School of Immunity and Infection and [2]School of Biosciences, University of Birmingham, Birmingham, UK.

[3]School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire, UK.

[†] These authors contributed equally to this work.

The authors declare no conflict of interest.

This article contains supporting information.

* For correspondence:

Email: D.F.Browning@bham.ac.uk
Email: I.R.Henderson@bham.ac.uk

I. R. Henderson, School of Immunity and Infection, University of Birmingham, Birmingham, B15 2TT, UK.

Tel: 0121-414-4368 Fax: 0121-414-3599

Abstract

Escherichia coli has been the leading model organism for many decades. It is a fundamental player in modern biology, facilitating the molecular biology revolution of the last century. The acceptance of *E. coli* as model organism is predicated primarily on the study of one *E. coli* lineage; *E. coli* K-12. However, the antecedents of today's laboratory strains have undergone extensive mutagenesis to create genetically tractable offspring but which resulted in loss of several genetic traits. We wished to determine whether these genetic lesions altered the physiology of *E. coli* such that observations made for *E. coli* K-12 were not reflective of the true physiology of the species. Here we have repaired the *wbbL* locus, restoring the ability of *E. coli* K-12 strain MG1655 to express the O antigen on its cell surface. We demonstrate that O-antigen production results in drastic alterations of many phenotypes and the density of the O antigen is critical for the observed phenotypes. Importantly, the presence of the O antigen enables laboratory strains of *E. coli* to enter the gut of the *Caenorhabditis elegans* nematode worm and to kill *C. elegans* at rates similar to pathogenic bacterial species. We show that killing is associated with bacterial resistance to mechanical shear and persistence in the *C. elegans* gut. We demonstrate *C. elegans* killing is a feature of other commensal *E. coli* and that killing occurs at the same rate as known pathogens . These results suggest *C. elegans* is not an effective model of human infectious disease.

Introduction

The outer membranes of Gram-negative bacteria function as a barrier to protect cells from toxic compounds such as antibiotics and detergents. The inner leaflet of the outer membrane is composed of phospholipids, whilst the outer leaflet is predominantly lipopolysaccharide (LPS). LPS consists of lipid A to which sugar units are added to generate the core LPS. The core LPS is further modified by the attachment of a repeat oligosaccharide unit, the O antigen (Raetz & Whitfield, 2002). In *Escherichia coli* the enzymes responsible for O antigen biosynthesis are encoded by the *rfb* cluster.

E. coli K-12 is considered to be the archetypal *E. coli* isolate and the premier model organism. Since its isolation in 1922, *E. coli* K-12 has become the workhorse of molecular biology. During this time it has been repeatedly passaged and has been subjected to ionising radiation, ultraviolet light and mutagens, resulting in a number of genetic lesions and an organism which has lost the F plasmid, bacteriophage λ and the ability to produce many surface-associated structures (Hobman *et al.*, 2007, Bachmann, 2004). Indeed, all strains of *E. coli* K-12 characterised to date are phenotypically rough, being unable to synthesize O antigen, due to mutations within the *rfb* locus (Fig. 1). In most *E. coli* K-12 strains this is due to the disruption of the *wbbL* gene by an IS*5* element, termed the *rfb-50* mutation. By complementing this lesion with plasmid vectors, Reeves and colleagues demonstrated that *wbbL* encoded a rhamnose transferase and *E. coli* K-12 was capable of synthesising O16 serotype LPS (Stevenson *et al.*, 1994, Liu & Reeves, 1994).

We hypothesised that the genetic lesions arising from years of laboratory growth and treatment with mutagens have resulted in a strain which does not reflect the biology of the species. Thus, interpretations from the study of *E. coli* K-12 and the acceptance of *E. coli* K-12 as a model organism could be flawed. Indeed, as more *E. coli* genomes have been sequenced it is clear that the laboratory adapted *E. coli* K-12 is far from typical and critically, previous phenotypic comparisons of *E. coli* K-12 with naturally occurring pathogenic and commensal strains have revealed it is ill-suited to life within the human gut or outside of the lab (Hobman et al., 2007, Anderson, 1975, Smith, 1975). To address our hypothesis we have regenerated the wild-type chromosomal *rfb* cluster in a strain of *E. coli* K-12 and determined how O antigen production affects its ability to survive hostile environments such as those encountered by wild-type strains of *E. coli*. Our results show that O antigen production increases the resistance of *E. coli* K-12 to many environmental insults. Importantly, we demonstrate that elaboration of the O antigen renders *E. coli* K-12 pathogenic in an accepted model of infection.

Results

Regeneration of the O antigen biosynthesis cluster of *E. coli* K-12. The *E. coli* K-12 strain MG1655 was the first *E. coli* strain to be sequenced and inspection of its *rfb* locus indicates that it contains an IS*5* insertion within *wbbL* (Blattner *et al.*, 1997) (Fig. 1). To confirm that other strain-specific mutations are not responsible for the inability of *E. coli* MG1655 to produce O antigen, we complemented this mutation by cloning an intact version of *wbbL* into the pET20b vector. Fig. 1B shows a silver stained SDS PAGE gel of total cellular protein and LPS and demonstrates that expression of the O antigen is restored in MG1655 by plasmid pET20b/ *wbbL* but not by the empty vector or when *wbbL* was inactivated by a single base pair deletion (*i.e.* pET20b/ *wbbL*Δ1). Thus, the inability of MG1655 to produce O antigen is due solely to the disruption of *wbbL*.

As plasmid complementation can lead to off-target phenotypes due to loss of plasmid maintenance, increased metabolic burdens or the presence of antibiotics to maintain the plasmid, we sought to regenerate the *rfb* cluster on the chromosome. This was achieved using the suicide vector pJP5603/ *wbbL*, which was transferred to MG1655 by conjugation. As pJP5603/ *wbbL* cannot stably replicate in MG1655, transconjugants can only arise by the integration of the plasmid into the chromosomal copy of *wbbL*. Integration of the plasmid upstream of the IS*5* element regenerates the *rfb* cluster, whilst integration downstream does not (Fig. 1C). This led to the generation of two strains, *E. coli* DFB1655 L9, which carries an intact *rfb* cluster, and *E. coli* DFB1655 L5 which carries *wbbL* but maintains the original IS*5* disruption within *wbbL*. Silver staining for LPS confirmed that both strains synthesized O antigen (Fig. 1D), however, DFB1655 L9 produced considerably more O antigen containing LPS than DFB1655 L5.

Research from other groups has shown that O antigen production leads to increased resistance to certain bacteriophages and a decreased ability to be transformed by recombinant plasmids (Ho & Waldor, 2007, Rubires *et al.*, 1997, Stern *et al.*, 1999). Our data show that DFB1655 L9 is resistant to phage P1 in phage cross-streak experiments (Fig. S1A) and has decreased transformation efficiency (Fig. S1B) when compared with the strains MG1655 and DFB1655 L5. The O antigen protects cells from the bactericidal action of complement (Rubires et al., 1997, Grossman *et al.*, 1987). To confirm that the restoration of O antigen biosynthesis affects the ability of MG1655 to survive complement-mediated killing, we exposed our strains to undiluted human serum and assessed their viability over 3 h. Data in Fig. S1C shows that all MG1655 and DFB1655 L5 cells were killed within 45 minutes but that DFB1655 L9 was

resistant to serum killing. Treating the serum with EGTA, so that only the adaptive component of serum-mediated killing was active, resulted in MG1655 being killed with 3 h while DFB1655 L9 cells remaining resistant to serum killing (Fig. S1D). These data indicate that DFB1655 L9 possesses an O antigen which functions in a manner similar to that of wild-type strains of *E. coli* and displays phenotypes previously reported for *E. coli* K-12 on restoration of O-antigen expression. Importantly, DFB1655 L5, which produces less O antigen, behaved similarly to the parental strain MG1655, indicating that the density of O antigen production is critical.

O antigen production does not affect growth of *E. coli* K-12. The biosynthesis of O antigen repeats and their incorporation onto the cell surface could place an additional energetic burden on the cell, affecting growth rates and the integrity of the outer membrane. To investigate this we examined the growth of each strain in Luria-Bertani broth (LB broth). No difference in growth rates could be distinguished between MG1655, DFB1655 L5 and DFB1655 L9 (Fig. S2A). To investigate this further, we examined the growth of MG1655 and DFB1655 L9 in the Biolog Phenotype Microarray (PM) (Bochner *et al.*, 2001, Zhou *et al.*, 2003) which compares the metabolic activity of each strain under ca. 2000 different growth conditions and in the presence of many antibiotics. The growth rate for DFB1655 L9 was found to indistinguishable from MG1655 for the majority of conditions tested. Differences were noted for only four conditions; when compared to MG1655, DFB1655 L9 was found to be slightly more resistant to the antifungal Tolylfluanid and the antiviral Trifluorothymidine but slightly more sensitive to the cationic detergents poly-L-lysine and the fatty acid biosynthesis inhibitor triclosan (McMurry *et al.*, 1998, Schweizer, 2001) (Table S1). MG1655 and DFB1655 L9 grown in liquid culture for 90 minutes and challenged with different triclosan concentrations confirmed DFB1655 L9 was mildly inhibited by Triclosan (Fig. S2B) confirming the veracity of the PM data.

The integrity of the outer membrane was assessed by a variety of techniques. First, Western blotting determined that the levels of the major outer membrane porins OmpF, OmpC and OmpA and the essential outer membrane lipoprotein BamD (Fig. S3A) were indistinguishable when DFB1655 L9 was compared to MG1655. Next, we examined the accumulation of the dye Hoechst 33342, a substrate of the major AcrAB-TolC efflux system in the presence and absence of the efflux pump inhibitor phenyl-arginine-β-naphthylamide (PAβN) (Coldham *et al.*, 2010, Webber *et al.*, 2008). Data in Fig. S3B shows that accumulation of Hoechst 33342 was similar in all strains, indicating chemical efflux was unaltered by the presence of the O

antigen. Finally, we used propidium iodide (PI) and Bis-(1,3-dibutylbarbituric acid) trimethine oxonol (BOX) staining to investigate cell viability and the integrity of the cell envelope. In each case, the O antigen-producing strains behaved similarly to MG1655 (Fig. S3C). Therefore, O antigen production does not affect growth, affect cell viability or substantially perturb outer membrane biogenesis under standard laboratory conditions.

O antigen production enhances killing of *C. elegans*. Previous experiments have demonstrated that *E. coli* K-12 is unable to colonise the intestinal tract of humans and animals. Furthermore, *E. coli* K-12 is often used as a negative control in models of pathogenicity. We wished to determine if restoration of the O antigen had an impact on the ability of *E. coli* to colonise the intestinal tract and/or mediate disease. The hermaphroditic worm *C. elegans* is often used as a model for microbial pathogenicity. Therefore, we investigated the ability of each of our strains to colonise and kill *C. elegans* using a slow kill assay, in which nematodes are fed bacteria and their viability monitored over 10 days (Aballay *et al.*, 2003). Results in Fig. 2A show that DFB1655 L9 killed *C. elegans* more rapidly than MG1655, DFB1655 L5 and the normal food source *E. coli* OP50 (Brenner, 1974). Importantly, DFB1655 L9 killed *C. elegans* at a rate almost identical to the pathogenic strain *Pseudomonas aeruginosa* PA14 (Fig. 2A). Previous investigations have demonstrated that the growth medium may alter the rate at which *C. elegans* succumbs to bacterial infection. To investigate if this was true for *E. coli* K-12 strains we repeated the above experiments on BHI agar plates. As reported previously, when grown on BHI agar *C. elegans* becomes more susceptible to the normal food source *E. coli* OP50 (Garsin *et al.*, 2001). Whilst MG1655 and DFB1655 L5 are more pathogenic for *C. elegans* when grown on BHI plates, importantly, DFB1655 L9 kills *C. elegans* significantly faster than the these strains ($p<0.05$) (Fig. 2B). These data indicate that O antigen production increases the virulence of the laboratory strain MG1655 and that despite decades of laboratory growth it has retained the ability to kill *C. elegans*.

As *E. coli* K-12 is derived from a commensal strain of *E. coli*, these experiments suggest other commensal strains of *E. coli* might have the capacity to kill *C. elegans*. To test this, we investigated the ability of the prototypical commensal strain *E. coli* HS (Rasko *et al.*, 2008) to kill *C. elegans*. Killing occurred at a rate similar to DFB1655 L9 and *P. aeruginosa* PA14 (Fig 2). We wished to determine if the dynamics of killing differed between commensal and pathogenic *E. coli* strains and whether the O antigen is also an important factor in nematode killing for other *E. coli* strains. To test this we examined the ability of enteroaggregative *E. coli* strain 042, a known human pathogen (Chaudhuri *et al.*, 2010), and *E. coli* DFB042, an

isogenic mutant derivative lacking O antigen (Fig. S4), to kill *C. elegans*. Results detailed in Fig. 2C show that *E. coli* 042 killed *C. elegans* at a rate similar to DFB1655 L9 and the commensal *E. coli* HS. Furthermore, *E. coli* DFB042 killed *C. elegans* more slowly ($p<0.05$). These data, indicate that *C. elegans* is susceptible to both commensal and pathogenic *E. coli* and confirm that in this model the O antigen is an important virulence factor for *E. coli*.

Killing is associated with persistent colonisation of the *C. elegans* intestine. Many species of bacteria elicit killing of *C. elegans* after colonisation of the nematode intestine (Marsh & May, 2012). To determine if MG1655, or its O antigen producing derivatives, could colonise the *C. elegans* gut, worms were grown on strains expressing Gfp and examined by fluorescent microscopy. Data in Fig. 3A show that DFB1655 L9 was detected throughout the intestines of infected worms, whilst DFB1655 L5 colonised poorly and MG1655 was not detected at all.

Several bacterial species are unable to persistently colonise the nematode gut and are rapidly expelled from the intestine (Lee *et al.*, 2011, Sifri *et al.*, 2003). To further investigate colonisation of the intestine, pulse-chase experiments, in which nematodes were fed each strain for one day and then transferred to plates containing its usual food source *E. coli* OP50 were done. Worms exposed to DFB1655 L9 still died more rapidly when compared to MG1655 or DFB1655 L5 (Fig. 3B). These data reveal that nematodes are unable to recover from the lethal effects of DFB1655 L9 once they have been in contact with the bacteria. To explore this further, we investigated gut colonisation of worms that were first fed on either Gfp-labelled MG1655 or DFB1655 L9 before being transferred to plates containing either non-fluorescent *E. coli* OP50 or non-fluorescent *E. coli* 042. Notably, after several days fluorescent bacteria could still be detected throughout the nematode alimentary canal of DFB1655 L9 but not MG1655 (Fig. 3C) demonstrating that DFB1655 L9 proliferates within and persistently colonises the worm intestine.

During the course of the latter experiments we noted worms fed on DFB1655 L9 showed distension of the alimentary canal when compared with worms fed MG1655 (Fig 4). Comparison of measurements of the hind gut of nematodes revealed those fed DFB1655 L9 were significantly more distended than those fed MG1655 ($P<0.01$). Importantly, during the course of these experiments we observed a significant portion of the worms fed DFB1655 L9 ruptured and released their intestinal content (Fig 4C). Rupture of worms was never observed for those fed MG1655 or DFB1655 L5. These data are consistent with our killing assays and suggest DFB1655 L9 kills worms by directly colonising the alimentary canal, leading to gut distension and rupture of the intestine.

O antigen production decreases biofilm formation. Alteration of the surface properties of many bacteria has been shown to affect the ability of cells to aggregate and form biofilms (Lee *et al.*, 2010, Schembri *et al.*, 2004). Since DFB1655 L9 could not be displaced by moving *C. elegans* onto *E. coli* OP50 or *E. coli* 042 we hypothesised that the ability of DFB1655 L9 to kill *C. elegans* was related to an increased ability to form a biofilm. To determine if O antigen expression affects this property we tested the ability of MG1655 and our O antigen-producing strains to form biofilms on solid surfaces. In contrast to our initial hypothesis, crystal violet biofilm assays demonstrated that strain DFB1655 L9 was impaired in its ability to form biofilms on polystyrene surfaces when compared with MG1655 and DFB1655 L5 (Fig. S5A). To investigate this in a more complex environment we examined biofilm formation in a continuous flow chamber over 42 h (Wells *et al.*, 2008). Comparative analysis of biofilms revealed a significant decrease in bio-volume and substratum coverage for DFB1655 L9 when compared to MG1655 at both 24 and 42 h ($P<0.001$) (Fig. S5B). Although there was less bio-volume and substratum coverage for DFB1655 L5 compared to MG1655 this was not significant ($P>0.05$). Thus, it is unlikely that the presence of the O antigen increases the ability of DFB1655 L9 to form a biofilm within the *C. elegans* gut.

O antigen enables *E. coli* K-12 to survive mechanical shear. *C. elegans* is often used as a model to understand the response of the innate immune system to components of bacterial pathogens. LPS is known to be a potent stimulator of the innate immune system. Therefore, we sought to determine if the presence of the O antigen may directly affect immune signalling and thereby increase the ability of MG1655 to colonise the *C. elegans* gut. To test this, worms were grown on MG1655 in the presence of saturating levels of purified LPS containing an O-antigen. However, the presence of LPS did not alter the ability of MG1655 to kill *C.elegans* ($P=0.538$), suggesting that LPS mediated signalling to the innate immune system is not responsible for the ability of DFB1655 L9 to kill *C. elegans* (Fig. 2C).

C. elegans harbours 15 phylogenetically diverse lysozyme genes (McGhee, 2007). We hypothesised that the presence of an intact O antigen may confer enhanced resistance to lysozyme. To test this hypothesis we grew MG1655, DFB1655 L5 and DFB1655 L9 in the presence of 1 and 2 mg ml^{-1} lysozyme in both broth and plate culture. The presence of an intact O antigen did not increase the ability DFB1655 L9 to grow in the presence of lysozyme when compared to the other strains (Fig. S6). To be effective at killing *E. coli in vitro* lysozyme is often combined with EDTA. Investigation of growth in the presence of lysozyme and EDTA

revealed that as expected MG1655 was readily killed. However, the presence of the O antigen did not increase the survival of either DFB1655 L5 or DFB1655 L9 (Fig. S6).

In *C. elegans* the first step in bacterial digestion is physical damage to the bacterium inflicted by the pharyngeal grinder (McGhee, 2007). Visualisation of GFP-tagged MG1655, DFB1655 L5 and DFB1655 L9 revealed the presence of DFB1655 L9 in the intestine but a lack of MG1655 and severely diminished numbers of DFB1655 L5 (Fig. 3A). From these observations we hypothesised that presence of the O antigen protected DFB1655 L9 from the macerating effects of the grinder. To test this hypothesis we placed similar numbers of bacteria in test tubes with glass beads, and observed the viability of bacterial cells after prolonged shaking. Interestingly, DFB1655 L9 survived the physical assault of the glass beads better than MG1655, with ca. ten-fold more viable bacteria present at the end of incubation (Fig. 5). No difference in bacterial numbers was observed when these strains were grown in the absence of glass beads.

Thus, we hypothesise that the O antigen permits survival of the bacteria into the intestine. Once within the intestine one or more factors allow the organism to colonise the intestine and nematode death follows once bacterial numbers increase to a density which causes rupture of the intestinal cavity.

Discussion

E. coli has long been considered the preeminent model organism. However, decades of laboratory growth has resulted in off-spring with notable mutations and phenotypes that do not represent the true biology of the species. We hypothesised that restoration of these mutations would alter the phenotypic characteristics previously recorded for *E. coli* K-12. One such mutation, which was noted early, conferred loss of O antigen production (Lederberg, 2004). In Gram-negative bacteria mature LPS contains multiple O antigen repeats attached to the core oligosaccharide and these extend from the cell surface into the external environment (Raetz & Whitfield, 2002, Peterson *et al.*, 1986, West *et al.*, 2005). Production of the O antigen confers on Gram-negative bacteria the ability to survive hostile environments, colonise hosts and cause disease (Ho & Waldor, 2007, West et al., 2005, Nesper *et al.*, 2001). By regenerating the *rfb* cluster we restored expression of the native O16 serotype O antigen in MG1655 (DFB1655 L9). This strain possessed novel phenotypes such as resistance to physical trauma however, reassuringly it possesses previously reported phenotypes including resistance to serum mediated killing and reduced genetic tractability (Ho & Waldor, 2007, Rubires et al., 1997, Stern et al., 1999).

We found the rough strain MG1655 was more genetically tractable; O antigen-expressing DFB1655 L9 was more resistant to bacteriophage P1 and to transformation with plasmids. These phenotypes can be simply explained by the O antigen acting as a barrier; as the P1 receptor is the LPS core it is likely that O antigen obscures this region of LPS and prevents phage attachment and infection (Ho & Waldor, 2007). It has been suggested that loss of O antigen production was a result of *E. coli* K-12 adaptation to laboratory life (Hobman et al., 2007). Indeed, loss of O antigen production by a similar IS-inactivation mechanism is a common occurrence for laboratory strains since the *rfb* clusters of both the *E. coli* B and C laboratory strains, which have different evolutionary lineages to *E. coli* K-12, are also disrupted by IS elements (Jeong *et al.*, 2009, Michel *et al.*, 2010, Crossman *et al.*, 2010). Thus, it is tempting to speculate that the reason that rough *E. coli* laboratory strains predominate is due to selection by early microbiologists because of their ease of manipulation.

An additional reason for the wide scale adoption of *E. coli* as a model organism was the inability of the bacterium to survive the killing activity to serum, thus providing assurance of its inability to harm people (Lederberg, 2004). The O antigen confers serum resistance by preventing the complement membrane attack complex (C5b-C9 complex) from gaining access to the bacterial outer membrane (Joiner *et al.*, 1982). Interestingly, while DFB1655 L9 was resistant to serum-mediated killing DFB1655 L5 was sensitive, even though it produces an O antigen. Previously, it was noted that *Salmonella enterica* serovar Montevideo cells are serum resistant provided ~20% of LPS molecules have more than 14 O antigen repeats per LPS molecule (Grossman et al., 1987). Thus, it is likely that a minimum density of O antigen is also required for MG1655 to resist serum killing.

Whilst reassuring that *E. coli* K-12 strains lacking O antigen were non-pathogenic for humans this observation represents an anthropocentric bias. Bacteria have evolved through a far longer battle with other micro-organisms and invertebrates. Indeed, nematodes such as *C. elegans*, began feasting on bacteria long before the appearance of mammals or man. Therefore, bacterial mechanisms to subvert nematode feeding behaviour must be of equally ancient provenance and would be expected to be present in strains of bacteria that are commensals in humans (Hobman et al., 2007). Our results show that O antigen expression enables *E. coli* K-12 to kill the nematode *C. elegans* more rapidly than MG1655 and enhances colonisation of the nematode intestine (Tan *et al.*, 1999). Importantly, *C. elegans* is maintained on *E. coli* OP50 in the laboratory; this strain is an *E. coli* B derivative that also lacks its O antigen (Jeong et al., 2009). *C. elegans* has been used as a model organism for studying the virulence of human pathogens

such as *E. coli* (Darby, 2005, Marsh & May, 2012). Indeed, wild-type pathovars of *E. coli*, presumably producing their native O antigens, have demonstrated faster killing times for *C. elegans* than the laboratory strains *E. coli* OP50 and MG1655 (Hwang *et al.*, 2010, Mellies *et al.*, 2006). We have recapitulated this observation here; the prototypical enteroaggregative *E. coli* strain 042 killed *C. elegans* more rapidly than the laboratory strains. Previously, Aballay *et al*. demonstrated that the O antigen from *S. enterica* serovar Typhimurium is also important for the colonisation and killing of *C. elegans*. Based on these observations, we propose that the O antigen protects the bacterium within the nematode gut and enables cells to colonise and multiply, establishing a persistent and lethal infection. This observation is supported by pulse-chase experiments, since once bacteria have entered the gut they are not displaced by pathogenic or non-pathogenic bacteria and the worms succumb to infection soon after. The results contrast with experiments that show other bacterial pathogens are unable to stably colonise the *C. elegans* intestine (Lee *et al.*, 2011, Sifri *et al.*, 2003).

We sought to identify how the O antigen might allow DFB1655 L9 to enhance nematode killing. When *C. elegans* feeds, bacteria are ingested via the mouth, concentrated and passed through a specialised pharyngeal organ termed the grinder eventually reaching the intestinal lumen. The grinder macerates the bacterial cells causing physical damage before the bacteria are digested within the intestines. Observations with GFP-labelled bacteria demonstrate that MG1655 does not persist once it enters the anterior intestine but that DFB1655 L9 persists colonising the entire length of the intestine. This suggests that MG1655 have not survived the grinder or have succumbed to the antimicrobial defences. Increased survival of DFB1655 L9 in the presence of the physical insult induced by growth with glass beads suggests that the presence of the O antigen protects bacterial cells as they pass through the grinder, with the O antigen acting as a ‘molecular shock absorber’. Once through the grinder the bacteria enter the anterior intestine which contains a number of innate defence mechanisms including production of enzymes such as lysozymes which degrade bacterial cell walls. Our experiments revealed no role for lysozyme in killing of MG1655, or indeed for ca. 2000 different growth conditions as assessed by PM analyses.

However, the above observations do not establish why the nematodes die after infection with DFB1655 L9. Observations of GFP-labelled bacteria suggest that DFB1655 L9 is better able to colonise the nematode gut and that this may deprive the worm of vital nutrients. Investigations with *Enterococcus faecium,* which heavily colonizes the intestines of *C. elegans,* reveal no increase in nematode mortality indicating that the worm is capable of deriving essential

nutrients even in the presence of colonising bacteria (Garsin *et al.*, 2003). Our results show that biofilm formation is impaired by O antigen, a phenomenon also seen in *Bradyrhizobium japonicumi* (Lee et al., 2010). The implication must therefore be that specific pathogenic mechanisms are required to adhere to the worm intestine; indeed *E. coli* K-12 has a plethora of fimbrial operons and protein secretion systems that have yet to be functionally characterised. Furthermore, recent observations have suggested *E. coli* can elaborate a toxin which results in *C. elegans* death (Anyanful *et al.*, 2005). Recall that due to evolutionary pressures factors required for nematode killing may be present in commensal bacteria and may differ from those required for human disease. In summary, based on the frequent observation of worms with ruptured intestines when fed on DFB1655 L9, it is likely that O-antigen production allows bacteria to survive passage through the grinder and once in the nematode gut a specific surface factor allows avid adhesion of the bacterium to the intestine, establishing a persistent infection and where increasing bacterial numbers lead to intestinal distension and eventual rupture, resulting in worm death.

In conclusion, two major findings can be derived from this study. First, the O antigen density is critically important for a variety of phenotypes and when creating genetic mutations researchers must be cognisant of the fact that perturbations in O antigen density can have a major impact on phenotype. Secondly, as *E. coli* K-12 and HS are not considered to be pathogenic organisms, our results directly question whether *C. elegans* is a suitable model for studying the virulence of pathogenic *E. coli*. Finally, since its isolation in 1922 *E. coli* K-12 has also lost the ability to produce O antigen, capsule, has been cured of the F plasmid and phage lambda, and has accumulated numerous other deleterious mutations (Hobman et al., 2007, Bachmann, 2004, Peleg *et al.*, 2005). These alterations have had a profound impact on *E. coli* K-12 biology and suggest that our heavy reliance on *E. coli* K-12 as a model organism may be ill-founded.

Experimental Procedures

Bacterial strains, plasmids and DNA fragments. The bacterial strains, plasmids and primers used in this work are listed in Table S2. Standard methods for cloning and manipulating DNA fragments were used throughout (Sambrook & Russell, 2001). Strains were cultured on LB broth or agar with ampicillin (100 μg ml^{-1}) and kanamycin (50 μg ml^{-1}) where appropriate.

Plasmid and strain construction. The intact *wbbL* open reading frame was amplified from *E. coli* K-12 strain WG1 using the primers WbbL(NdeI) and WbbL(HindIII). Note that WG1 is an early isolate of *E. coli* K-12 (Fig. S1) (Liu & Reeves, 1994). The PCR amplicon was restricted

with *Nde*I and *Hin*dIII and cloned into pET20b to generate pET20b/ *wbbL*. Plasmid pET20b/ *wbbL*Δ1, which carries a single base pair deletion of *wbbL*, was fortuitously isolated during construction of pET20b/ *wbbL*. The *wbbL* PCR product was also cut with *Eco*RI and *Xho*I, cloned into the suicide vector pJP5603 restricted with *Eco*RI and *Sal*I. The resulting plasmid was maintained in DH5α λ*pir* cells (Penfold & Pemberton, 1992). To construct the O antigen-producing strains, DFB1655 L5 and DFB1655 L9, plasmid pJP5603/ *wbbL* was transferred from *E. coli* strain S17-1 λ*pir* by conjugation to MG1655 resulting in integration of pJP5603/ *wbbL* into the chromosomal *wbbL* locus of MG1655. The site of integration was verified by PCR using primers WbbLFW and M13Rev and PCR products were sequenced. O antigen biosynthesis in enteroaggregative *E. coli* strain 042 (Chaudhuri et al., 2010) was disrupted by amplifying a 719 bp internal PCR fragment of *wbaC* using primers wbaCUP and wbaCDWN. The PCR amplicon was restricted with *Xba*I and cloned into the suicide plasmid pCVD442. Plasmids derived from pCVD442 were maintained in DH5α λ*pir* cells. pCVD432/ *wbaC* was transferred from S17-1 λ*pir* to *E. coli* 042 by conjugation and its integration into the chromosomal *wbaC* gene checked by PCR. Plasmid pJB42 was derived from pDOC-C (Lee *et al.*, 2009). The region between *I-SceI* target sites was replaced by a fragment of DNA containing the *lac* promoter region (-92 bp to +122 bp relative to the transcription start site) which was fused to the fluorescent reporter gene emerald GFP.

Detection of bacterial components. Analysis of total protein and LPS samples was carried out as previously described (Browning *et al.*, 2003, Knowles *et al.*, 2011). Measured numbers of viable bacteria were resuspended in SDS lysis buffer (0.1 M Tris (pH 5.8), 0.2 M β-mercaptoethanol, 20% glycerol and 2% SDS) and heated to 95°C for 3 mins generating samples containing total protein and LPS. To generate LPS only containing samples, 50 µl of each sample was mixed with 25 µl of 1 mg ml^{-1} proteinase K (Qiagen) and incubated at 60°C for 1 h. Samples were resolved by SDS-PAGE and visualised using a SilverQuest Silver Staining Kit (Invitrogen). OmpF (Bennion *et al.*, 2010) and BamD (Rossiter *et al.*, 2011) proteins were detected using antiserum raised in rabbits, and the α subunit of RNA polymerase was detected using mouse monoclonal antibodies (Neoclone). Blots were developed using the ECL Plus Western Blotting Detection System (GE Healthcare).

Nematode virulence assays. *C. elegans* strain Bristol N2 was cultured with *E. coli* strain OP50 using standard methods and survival assays were performed as before (Aballay et al., 2003). Larval stage 4 *C. elegans* were picked and transferred onto assay plates, with 20 worms on each plate. Plates were incubated at 25°C and scored daily for survival. Worms were

regularly transferred onto a fresh NGM plate containing the same bacteria from the same original culture during the fertile period. A Kaplan Meier estimate was used to determine the probability of *C. elegans* survival. Survival curves were generated by plotting probability of survival against time and were then compared using the log rank test to establish differences between two curves. To examine the effect of LPS on nematode survival 200 μg purified LPS was spotted onto the bacterial inoculum on agar plates and worm scored daily for survival.

Phenotypic analyses. To test for phage P1 resistance, strains were cross-streaked against a P1 lysate as before (Ho & Waldor, 2007). Biofilm formation was examined on polystyrene surfaces using 96-well microtitre plates as before (Raghunathan *et al.*, 2011). Flow chamber experiments were performed as before (Wells et al., 2008) and biofilms were formed on glass surfaces in a multichannel flow system. Strains were transformed with the GFP-expressing plasmid pJB42. Biofilm development was monitored using a confocal scanning laser microscopy. Appropriate z-stacks were collected for each strain and analyzed by using the COMSTAT software program (Heydorn *et al.*, 2000). Biofilm experiments were performed in triplicate. Serum killing assays were performed as before (Raghunathan et al., 2011) with viable counts determined after 45, 90 and 180 min. For EGTA experiments, EGTA and $MgSO_4$ was added to the serum at 10mM and 5mM concentrations respectively before incubating with bacteria. Experiments were performed in triplicate. Phenotype microarray growth was performed by Biolog Inc. (Hayward, California USA) as described previously (Bochner et al., 2001, Zhou et al., 2003). Sensitivity to triclosan was investigated by examining the growth of strains in liquid culture. The optical density was monitored using a FluoStar Optima (BMG labtech, U.K). For each strain two biological replicate overnight cultures were used to inoculate four technical replicate cultures each and 200 μl of each culture added to separate wells of a microtitre tray which was then incubated at 37°C with shaking (Andrews, 2001). Triclosan was injected after 90 min incubation. Membrane permeability and efflux activity were examined by measuring the accumulation of Hoechst 33342. Experiments were performed in the presence and absence of the efflux pump PAβN as previously described (Coldham et al., 2010, Webber & Coldham, 2010). Mechanical shearing was performed by incubating concentrated cultures (OD_{600}:10) with or without 12 glass beads. Tubes were shaken at 400 rpm for 12 h. Samples were taken from the tubes at 12 h for dilution and plating on LB agar. Following overnight CFUs were calculated and the difference between 0 and 12 h expressed as Log10 of CFU ml^{-1}. Experiments were performed in triplicate on two separate occasions. Lysozyme resistance was examined by growing strains to OD_{600}:0.6 in LB broth. Either 0, 1 or 2 mg ml^{-1} of lysozyme (Sigma) was added to the cultures and shaken at 37°C for 2 h before dilution and plating on LB

agar. After incubation CFU ml^{-1} were calculated. 1 mg ml^{-1} lysozyme with 10 mM EDTA was also added to cultures as a control. Experiments were performed in triplicate on three separate occasions.

Acknowledgements

This work was generously supported by funding from the Medical Research Council (DFB and IRH) and BBSRC to JLH. We thank Rajeev Misra for donating anti-OmpF antibodies.

References

Aballay, A., Drenkard, E., Hilbun, L.R.and Ausubel, F.M. (2003) *Caenorhabditis elegans* innate immune response triggered by *Salmonella enterica* requires intact LPS and is mediated by a MAPK signaling pathway. *Curr Biol* **13**: 47-52.

Anderson, E.S. (1975) Viability of, and transfer of a plasmid from, *E. coli* K12 in human intestine. *Nature* **255**: 502-504.

Andrews, J.M. (2001) Determination of minimum inhibitory concentrations. *J Antimicrob Chemoth* **48**: 5-16.

Anyanful, A., Dolan-Livengood, J.M., Lewis, T., Sheth, S., Dezalia, M.N., Sherman, M.A*., et al.* (2005) Paralysis and killing of *Caenorhabditis elegans* by enteropathogenic *Escherichia coli* requires the bacterial tryptophanase gene. *Mol Microbiol* **57**: 988-1007.

Bachmann, B.J. (2004) Derivations and genotypes of some mutant derivatives of *Escherichia coli* K-12. In: *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology. Washington: ASM, pp.

Bennion, D., Charlson, E.S., Coon, E.and Misra, R. (2010) Dissection of beta-barrel outer membrane protein assembly pathways through characterizing BamA POTRA 1 mutants of *Escherichia coli. Mol Microbiol* **77**: 1153-1171.

Blattner, F.R., Plunkett, G., Bloch, C.A., Perna, N.T., Burland, V., Riley, M*., et al.* (1997) The complete genome sequence of *Escherichia coli* K-12. *Science* **277**: 1453-1462.

Bochner, B.R., Gadzinski, P.and Panomitros, E. (2001) Phenotype microarrays for high-throughput phenotypic testing and assay of gene function. *Genome Res* **11**: 1246-1255.

Brenner, S. (1974) The genetics of *Caenorhabditis elegans*. *Genetics* **77**: 71-94.

Browning, D.F., Whitworth, D.E.and Hodgson, D.A. (2003) Light-induced carotenogenesis in *Myxococcus xanthus*: functional characterization of the ECF sigma factor CarQ and antisigma factor CarR. *Mol Microbiol* **48**: 237-251.

Chaudhuri, R.R., Sebaihia, M., Hobman, J.L., Webber, M.A., Leyton, D.L., Goldberg, M.D*., et al.* (2010) Complete genome sequence and comparative metabolic profiling of the prototypical enteroaggregative *Escherichia coli* strain 042. *PLoS One* **5**: e8801.

Coldham, N.G., Webber, M., Woodward, M.J.and Piddock, L.J. (2010) A 96-well plate fluorescence assay for assessment of cellular permeability and active efflux in *Salmonella enterica* serovar Typhimurium and *Escherichia coli*. *J Antimicrob Chemoth* **65**: 1655-1663.

Crossman, L.C., Chaudhuri, R.R., Beatson, S.A., Wells, T.J., Desvaux, M., Cunningham, A.F*., et al.* (2010) A commensal gone bad: complete genome sequence of the prototypical enterotoxigenic *Escherichia coli* strain H10407. *J Bacteriol* **192**: 5822-5831.

Darby, C. (2005) Interactions with microbial pathogens. *WormBook : the online review of C. elegans biology*: 1-15.

Garsin, D.A., Sifri, C.D., Mylonakis, E., Qin, X., Singh, K.V., Murray, B.E., *et al.* (2001) A simple model host for identifying Gram-positive virulence factors. *Proc Natl Acad Sci U S A* **98**: 10892-10897.

Garsin, D.A., Villanueva, J.M., Begun, J., Kim, D.H., Sifri, C.D., Calderwood, S.B., *et al.* (2003) Long-lived *C. elegans daf-2* mutants are resistant to bacterial pathogens. *Science* **300**: 1921.

Grossman, N., Schmetz, M.A., Foulds, J., Klima, E.N., Jimenez-Lucho, V.E., Leive, L.L., *et al.* (1987) Lipopolysaccharide size and distribution determine serum resistance in *Salmonella montevideo*. *J Bacteriol* **169**: 856-863.

Heydorn, A., Nielsen, A.T., Hentzer, M., Sternberg, C., Givskov, M., Ersboll, B.K., *et al.* (2000) Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology* **146**: 2395-2407.

Ho, T.D.and Waldor, M.K. (2007) Enterohemorrhagic *Escherichia coli* O157:H7 *gal* mutants are sensitive to bacteriophage P1 and defective in intestinal colonization. *Infect Immun* **75**: 1661-1666.

Hobman, J.L., Penn, C.W.and Pallen, M.J. (2007) Laboratory strains of *Escherichia coli*: model citizens or deceitful delinquents growing old disgracefully? *Mol Microbiol* **64**: 881-885.

Hwang, J., Mattei, L.M., VanArendonk, L.G., Meneely, P.M.and Okeke, I.N. (2010) A pathoadaptive deletion in an enteroaggregative *Escherichia coli* outbreak strain enhances virulence in a *Caenorhabditis elegans* model. *Infect Immun* **78**: 4068-4076.

Jeong, H., Barbe, V., Lee, C.H., Vallenet, D., Yu, D.S., Choi, S.H., *et al.* (2009) Genome sequences of *Escherichia coli* B strains REL606 and BL21(DE3). *J Mol Biol* **394**: 644-652.

Joiner, K.A., Hammer, C.H., Brown, E.J., Cole, R.J.and Frank, M.M. (1982) Studies on the mechanism of bacterial resistance to complement-mediated killing. I. Terminal complement components are deposited and released from *Salmonella minnesota* S218 without causing bacterial death. *J Exp Med* **155**: 797-808.

Knowles, T.J., Browning, D.F., Jeeves, M., Maderbocus, R., Rajesh, S., Sridhar, P., *et al.* (2011) Structure and function of BamE within the outer membrane and the beta-barrel assembly machine. *EMBO Rep* **12**: 123-128.

Lederberg, J., (2004) *E. coli* K-12. *Microbiol Today* **31**: 116.

Lee, D.J., Bingle, L.E., Heurlier, K., Pallen, M.J., Penn, C.W., Busby, S.J., *et al.*, (2009) Gene doctoring: a method for recombineering in laboratory and pathogenic *Escherichia coli* strains. *BMC Microbiol* **9**: 252.

Lee, S.H., Ooi, S.K., Mahadi, N.M., Tan, M.W.and Nathan, S. (2011) Complete killing of *Caenorhabditis elegans* by *Burkholderia pseudomallei* is dependent on prolonged direct association with the viable pathogen. *PLoS One* **6**: e16707.

Lee, Y.W., Jeong, S.Y., In, Y.H., Kim, K.Y., So, J.S.and Chang, W.S. (2010) Lack of O-polysaccharide enhances biofilm formation by *Bradyrhizobium japonicum*. *Lett Appl Microbiol* **50**: 452-456.

Liu, D.and Reeves, P.R. (1994) *Escherichia coli* K12 regains its O antigen. *Microbiology* **140**: 49-57.

Marsh, E.K.and May, R.C. (2012) *Caenorhabditis elegans*, a model organism for investigating immunity. *Appl Environ Microbiol* **78**: 2075-2081.

McGhee, J.D. (2007) The *C. elegans* intestine. *WormBook : the online review of C. elegans biology*: 1-36.

McMurry, L.M., Oethinger, M.and Levy, S.B. (1998) Triclosan targets lipid synthesis. *Nature* **394**: 531-532.

Mellies, J.L., Barron, A.M., Haack, K.R., Korson, A.S.and Oldridge, D.A. (2006) The global regulator Ler is necessary for enteropathogenic *Escherichia coli* colonization of *Caenorhabditis elegans*. *Infect Immun* **74**: 64-72.

Michel, A., Clermont, O., Denamur, E.and Tenaillon, O. (2010) Bacteriophage PhiX174's ecological niche and the flexibility of its *Escherichia coli* lipopolysaccharide receptor. *Appl Environ Microbiol* **76**: 7310-7313.

Nesper, J., Lauriano, C.M., Klose, K.E., Kapfhammer, D., Kraiss, A.and Reidl, J. (2001) Characterization of *Vibrio cholerae* O1 El *tor galU* and *galE* mutants: influence on lipopolysaccharide structure, colonization, and biofilm formation. *Infect Immun* **69**: 435-445.

Peleg, A., Shifrin, Y., Ilan, O., Nadler-Yona, C., Nov, S., Koby, S*., et al.* (2005) Identification of an *Escherichia coli* operon required for formation of the O-antigen capsule. *J Bacteriol* **187**: 5259-5266.

Penfold, R.J.and Pemberton, J.M. (1992) An improved suicide vector for construction of chromosomal insertion mutations in bacteria. *Gene* **118**: 145-146.

Peterson, A.A., Haug, A.and McGroarty, E.J. (1986) Physical properties of short- and long-O-antigen-containing fractions of lipopolysaccharide from *Escherichia coli* 0111:B4. *J Bacteriol* **165**: 116-122.

Raetz, C.R.and Whitfield, C. (2002) Lipopolysaccharide endotoxins. *Annu Rev Biochem* **71**: 635-700.

Raghunathan, D., Wells, T.J., Morris, F.C., Shaw, R.K., Bobat, S., Peters, S.E*., et al.* (2011) SadA, a trimeric autotransporter from *Salmonella enterica* serovar Typhimurium, can promote biofilm formation and provides limited protection against infection. *Infect Immun* **79**: 4342-4352.

Rasko, D.A., Rosovitz, M.J., Myers, G.S., Mongodin, E.F., Fricke, W.F., Gajer, P*., et al.* (2008) The pangenome structure of *Escherichia coli*: comparative genomic analysis of E. coli commensal and pathogenic isolates. *J Bacteriol* **190**: 6881-6893.

Rossiter, A.E., Leyton, D.L., Tveen-Jensen, K., Browning, D.F., Sevastsyanovich, Y., Knowles, T.J*., et al.* (2011) The essential beta-barrel assembly machinery complex components BamD and BamA are required for autotransporter biogenesis. *J Bacteriol* **193**: 4250-4253.

Rubires, X., Saigi, F., Pique, N., Climent, N., Merino, S., Alberti, S*., et al.* (1997) A gene (*wbbL*) from *Serratia marcescens* N28b (O4) complements the *rfb-50* mutation of *Escherichia coli* K-12 derivatives. *J Bacteriol* **179**: 7581-7586.

Sambrook, J.and Russell, D.W. (2001) *Molecular cloning: a laboratory manual*. CSHL Press, New York.

Schembri, M.A., Dalsgaard, D.and Klemm, P. (2004) Capsule shields the function of short bacterial adhesins. *J Bacteriol* **186**: 1249-1257.

Schweizer, H.P., (2001) Triclosan: a widely used biocide and its link to antibiotics. *FEMS Microbiol Lett* **202**: 1-7.

Sifri, C.D., Begun, J., Ausubel, F.M.and Calderwood, S.B. (2003) *Caenorhabditis elegans* as a model host for *Staphylococcus aureus* pathogenesis. *Infect Immun* **71**: 2208-2217.

Smith, H.W., (1975) Survival of orally administered *E. coli* K 12 in alimentary tract of man. *Nature* **255**: 500-502.

Stern, R.J., Lee, T.Y., Lee, T.J., Yan, W., Scherman, M.S., Vissa, V.D*., et al.* (1999) Conversion of dTDP-4-keto-6-deoxyglucose to free dTDP-4-keto-rhamnose by the *rmIC* gene products of *Escherichia coli* and *Mycobacterium tuberculosis*. *Microbiology* **145**: 663-671.

Stevenson, G., Neal, B., Liu, D., Hobbs, M., Packer, N.H., Batley, M*., et al.* (1994) Structure of the O antigen of *Escherichia coli* K-12 and the sequence of its *rfb* gene cluster. *J Bacteriol* **176**: 4144-4156.

583 Tan, M.W., Rahme, L.G., Sternberg, J.A., Tompkins, R.G.and Ausubel, F.M. (1999)
584 *Pseudomonas aeruginosa* killing of *Caenorhabditis elegans* used to identify *P.*
585 *aeruginosa* virulence factors. *Proc Natl Acad Sci U S A* **96**: 2408-2413.
586 Webber, M.A.and Coldham, N.G. (2010) Measuring the activity of active efflux in Gram-
587 negative bacteria. *Methods Mol Biol* **642**: 173-180.
588 Webber, M.A., Randall, L.P., Cooles, S., Woodward, M.J.and Piddock, L.J. (2008) Triclosan
589 resistance in *Salmonella enterica* serovar Typhimurium. *J Antimicrob Chemoth* **62**: 83-
590 91.
591 Wells, T.J., Sherlock, O., Rivas, L., Mahajan, A., Beatson, S.A., Torpdahl, M.*, et al.* (2008)
592 EhaA is a novel autotransporter protein of enterohemorrhagic *Escherichia coli*
593 O157:H7 that contributes to adhesion and biofilm formation. *Environ Microbiol* **10**:
594 589-604.
595 West, N.P., Sansonetti, P., Mounier, J., Exley, R.M., Parsot, C., Guadagnini, S.*, et al.* (2005)
596 Optimization of virulence functions through glucosylation of Shigella LPS. *Science*
597 **307**: 1313-1317.
598 Zhou, L., Lei, X.H., Bochner, B.R.and Wanner, B.L. (2003) Phenotype microarray analysis of
599 *Escherichia coli* K-12 mutants with deletions of all two-component systems. *J*
600 *Bacteriol* **185**: 4956-4972.
601
602

Fig. 1. Regeneration of the *E. coli* K-12 *rfb* O antigen biosynthetic locus.

A. Organisation of the *rfb* cluster from *E. coli* K-12 strain MG1655, showing the IS*5* inactivation of *wbbL* (*i.e.* the *rfb-50* mutation) and the approximate deletion of the *rfb* cluster in *E. coli* K-12 strain WG1 (*i.e.* the *rfb-51* mutation).

B. Strain MG1655, carrying either empty pET20b (lanes 2 and 5), pET20b/ *wbbL* (lanes 3 and 6) or pET20b/ *wbbL*Δ1 (lanes 4 and 7) were grown to mid logarithmic phase and total protein samples plus LPS (lanes 2 to 4) and LPS only samples (lanes 5 to 7) were prepared. Samples were separated using SDS PAGE and gels were silver stained.

C. Integration of the conjugative suicide vector pJP5603/ *wbbL* into the chromosome of MG1655. Integration upstream of the IS*5* element (recombination event 1) regenerates a complete *rfb* cluster (strain DFB1655 L9), whilst integration downstream of the IS*5* element (recombination event 2) does not (strain DFB1655 L5). Note that *gnd* is not part of the *rfb* cluster and thus the insertion of pJP5603/ *wbbL* will not cause polar effects on its expression. Silver stained SDS PAGE gels of total protein and LPS samples from *E. coli* K-12 strains.

D. Strains MG1655 (lanes 2 and 5), DFB1655 L5 (lanes 3 and 6) and DFB1655 L9 (lanes 4 and 7) were grown to mid logarithmic phase and total protein samples plus LPS (lanes 2 to 4) and LPS only (lane 5 to 7) were subjected to SDS PAGE and silver staining. In both panels SDS PAGE markers were loaded in lane 1.

Fig. 2. O-antigen biosynthesis enables *E. coli* K-12 to kill *C. elegans*.

A. Nematode slow-kill virulence assays on NGM agar. The ability of each strain to kill the nematode worm *C. elegans* was examined over a 10-day period. The LT_{50} (time for half of the worms to die) was calculated for each experiment: *E. coli* MG1655 LT_{50}=9.36; DFB1655 L5 LT_{50}=7.318 and DFB1655 L9 LT_{50}=4.52. The *E. coli* strain OP50, a commonly used as a food source for *C. elegans*, was used as a negative control (LT_{50}>10). The opportunistic pathogen *Pseudomonas aeruginosa* PA14 (LT_{50}=3.54), which was previously shown to kill *C. elegans*, was included as a positive control.

B. Killing of *C. elegans* was monitored during growth of MG1655 (LT_{50}=6.54), DFB1655 L5 (LT_{50}=6.42) and DFB1655 L9 (LT_{50}=4.72) on BHI agar. *E. coli* OP50 was included as a negative control (LT_{50}>10).

C. Nematodes were fed *E. coli* 042 (LT_{50}=4.09), DFB042, a mutant strain lacking O-antigen expression (LT_{50}=5.90), the commensal strain *E. coli* HS (LT_{50}=4.81) or MG1655 (LT_{50}=9.82). *C. elegans* killing was assessed. The effect of LPS on nematode survival was examined by feeding worms MG1655 supplemented with purified O-antigen containing LPS (LT_{50}=10).

Fig. 3. DFB1655 L9 colonises *C. elegans* intestine.

A. Confocal fluorescent microscopy images of GFP-expressing bacteria colonizing the *C. elegans* intestine. Young adult worms were fed bacterial strains MG1655 (i, iv), DFB1655 L5 (ii, v) and DFB1655 L9 (iii, vi). All strains carried the GFP-expressing plasmid pJB42 and panels iv to vi are enlargements of the boxed areas in panels i to iii, respectively.

B. Nematode worms were fed MG1655 (LT_{50}=9.92), DFB1655 L5 (LT_{50}>10) or DFB1655 L9 (LT_{50}=6.21) for one day and then transferred to plates containing the normal foodstuff *E. coli* OP50.

C. Fluorescent microscopy of (i) Gfp-labelled MG1655 and (ii) Gfp-labelled DFB1655 L9 colonisation of the worm intestine after being fed the strains for one day before transferring to plates containing *E. coli* 042 for three days.

Fig. 4. Colonisation of *C. elegans* by DFB1655 L9 leads to distension of the nematode gut.

A. Fluorescence microscope imaging of *C. elegans* fed with Gfp-labelled DFB1655 L9 or MG1655.

B. The hindguts of 20 worms fed with either MG1655 or DFB1655 L9 were measured. Gut width was calculated as a percentage of total worm width.

C. Fluorescent microscopy of a worm with ruptured intestines after colonisation with DFB1655 L9.

Fig. 5. O-antigen production protects *E. coli* from mechanical shearing.

Concentrated cultures were shaken at 400 rpm for 12 h in either the presence or absence of glass beads. Cultures were plated and CFU ml^{-1} difference between 0 h and 12 h expressed as Log10 CFU ml^{-1}. Thus, a value of -3 would indicate a 3 log drop in CFU ml^{-1} after 12 h.

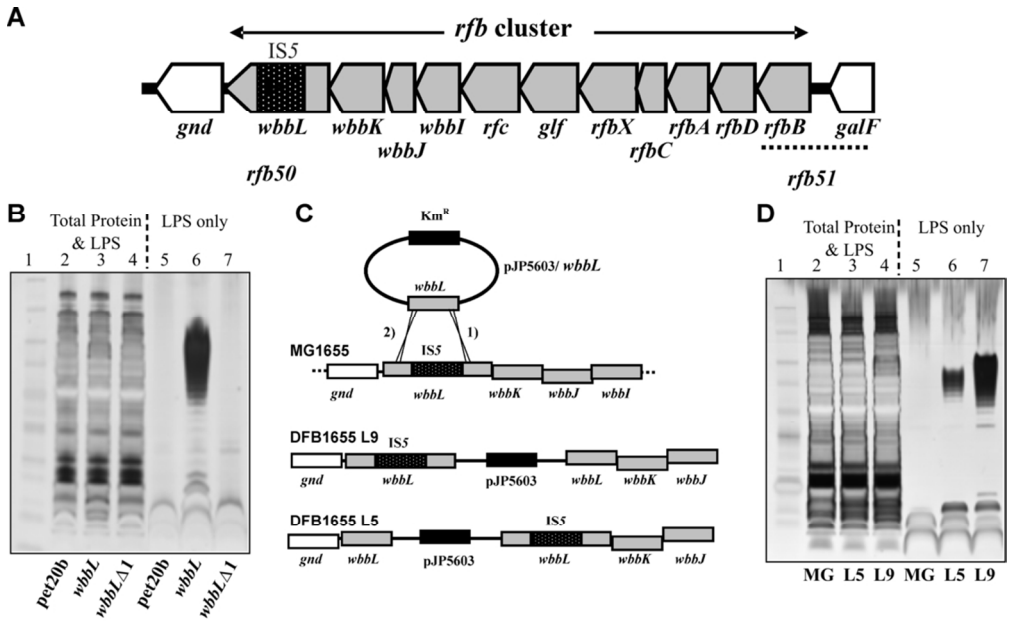


Fig. 1. Regeneration of the E. coli K-12 rfb O antigen biosynthetic locus
98x60mm (300 x 300 DPI)

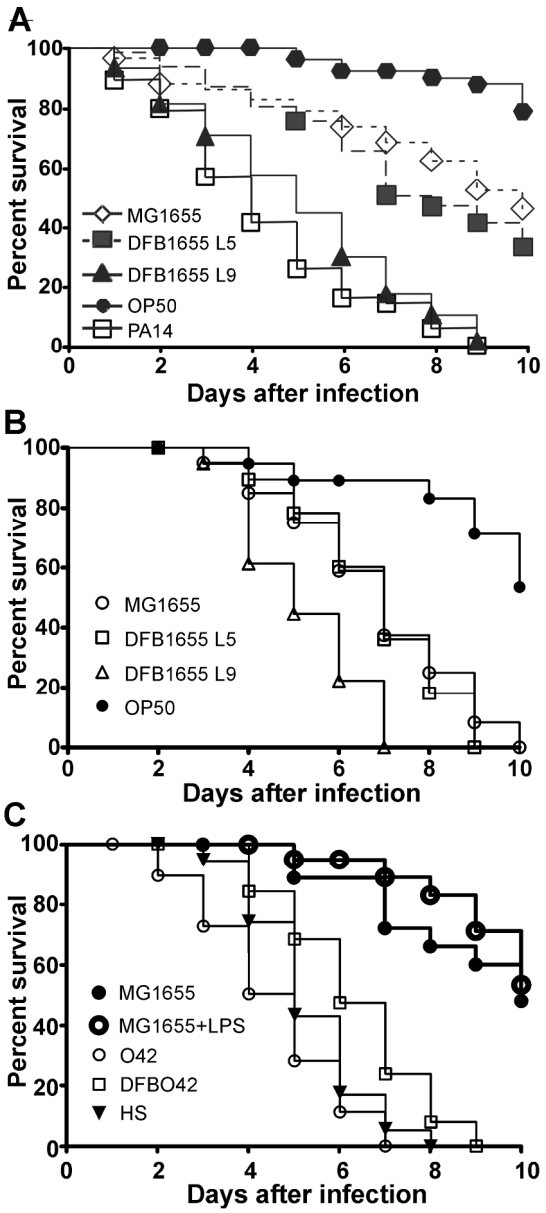


Fig. 2. O-antigen biosynthesis enables E. coli K-12 to kill C. elegans
152x340mm (300 x 300 DPI)

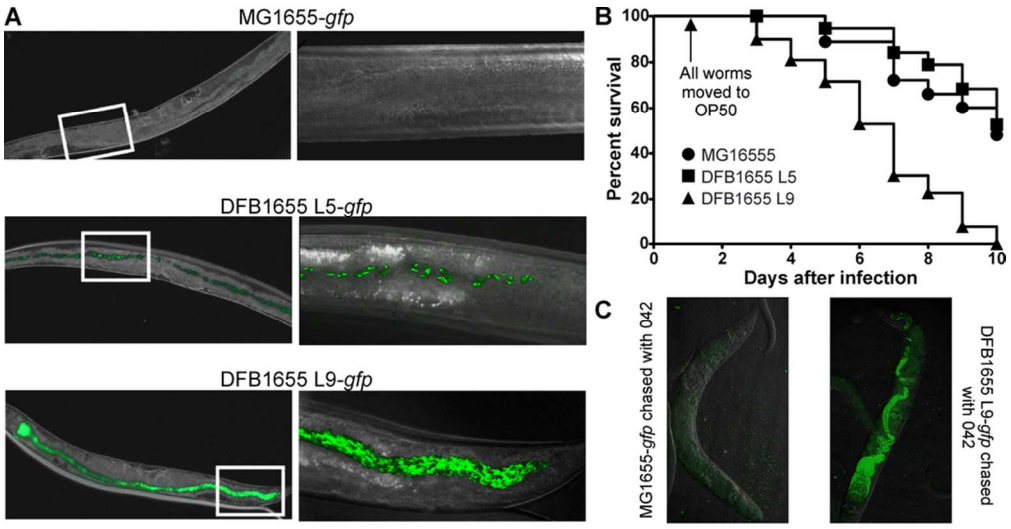


Fig. 3. DFB1655 L9 colonises C. elegans intestine
88x46mm (300 x 300 DPI)

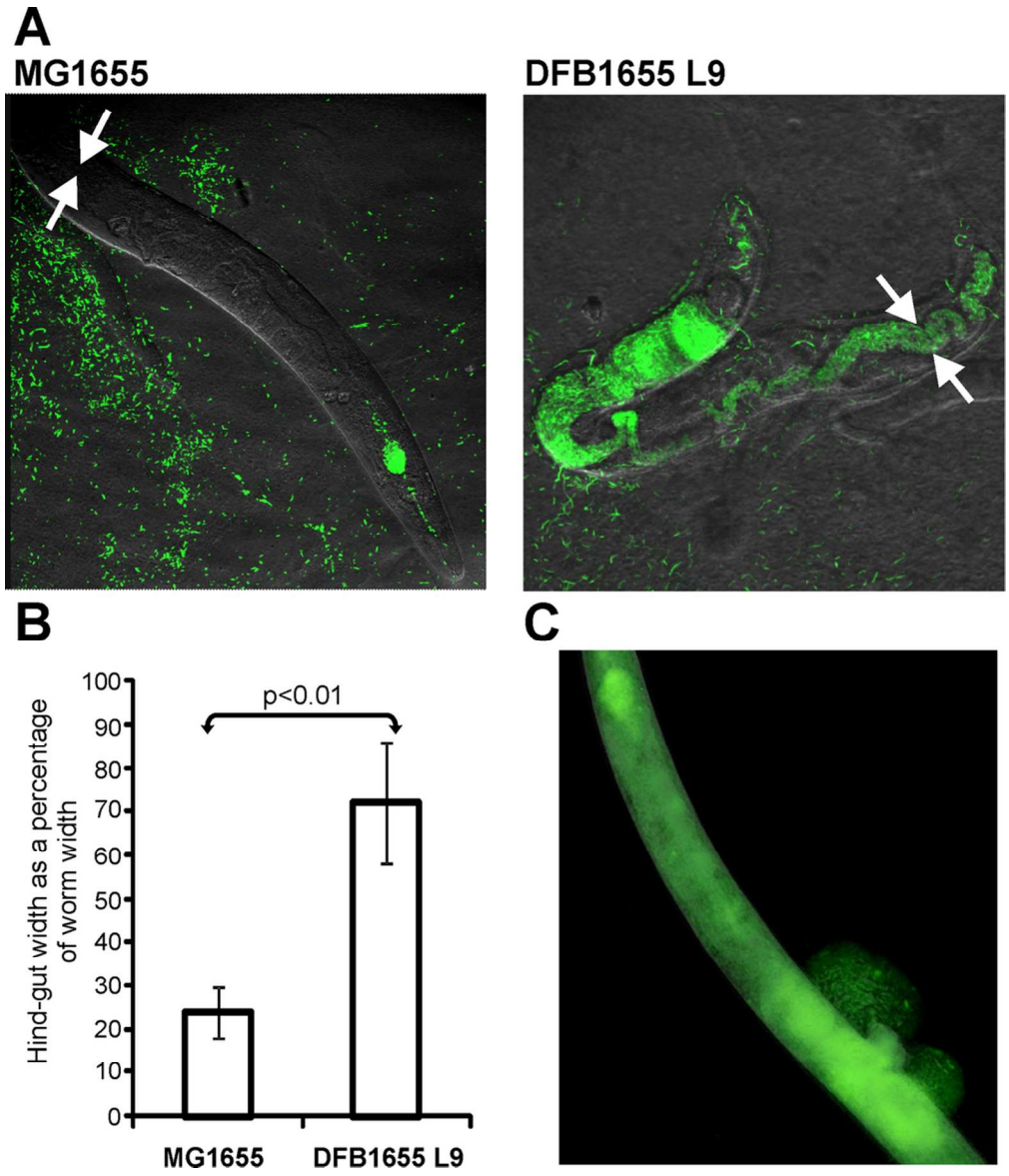


Fig. 4. Colonisation of C. elegans by DFB1655 L9 leads to distension of the nematode gut.
92x107mm (300 x 300 DPI)

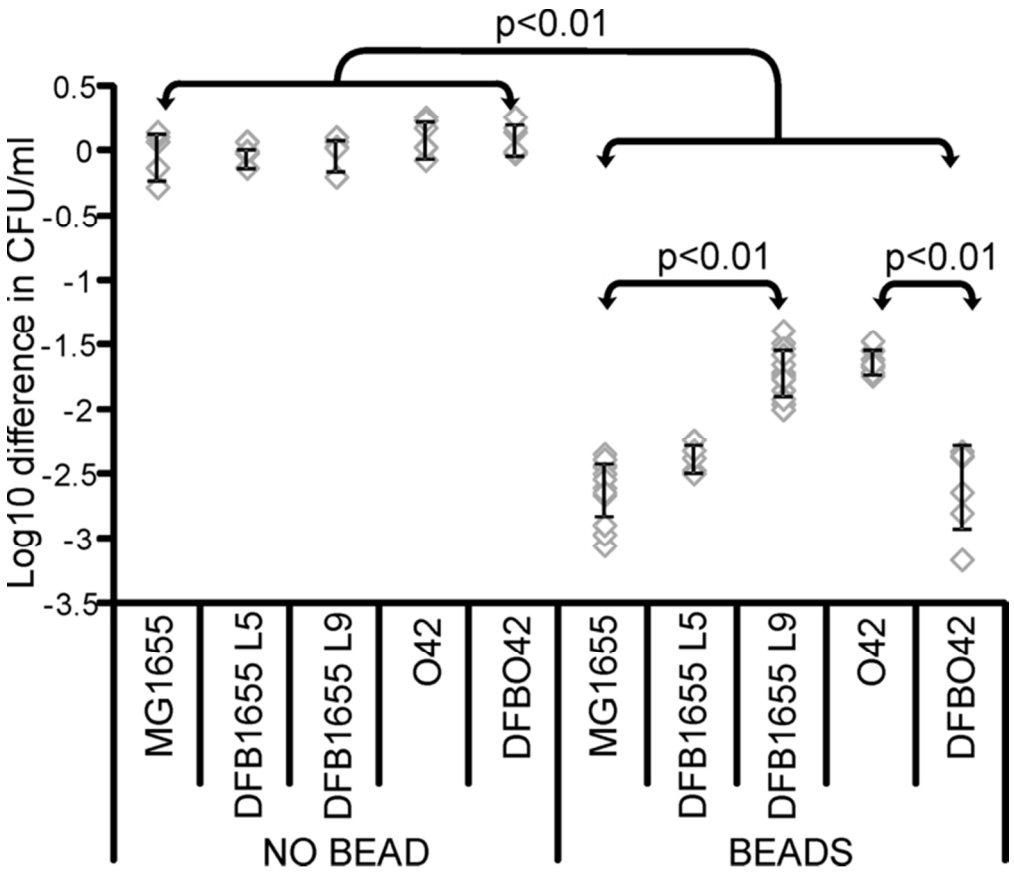
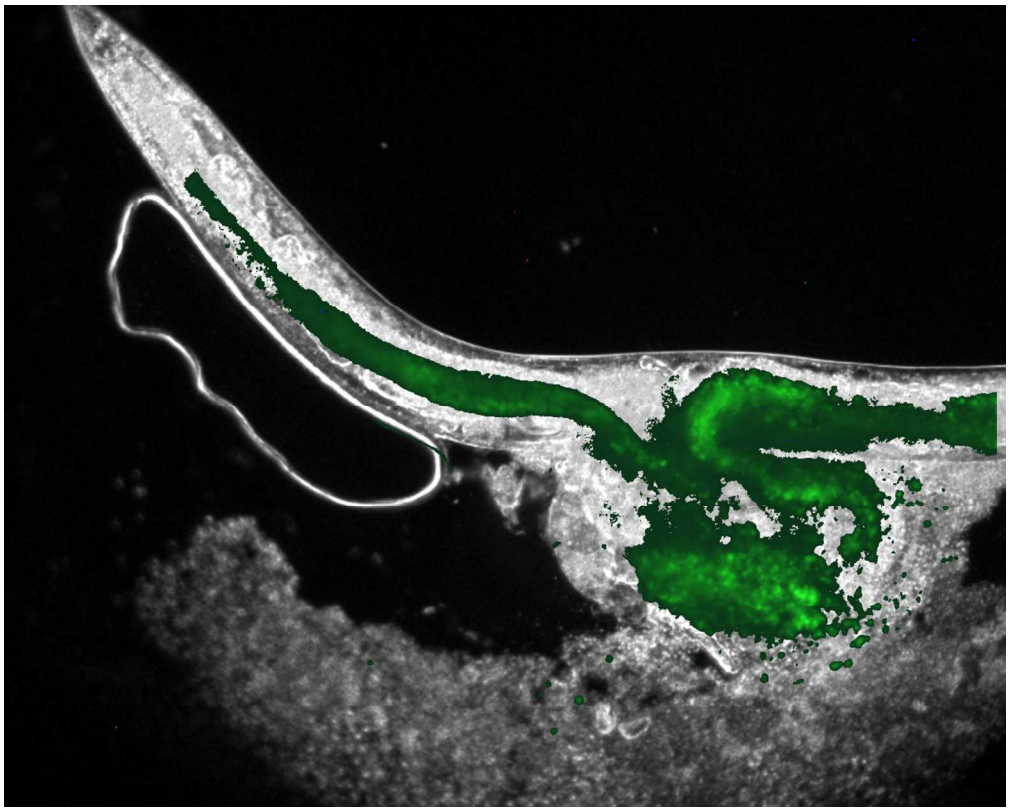


Fig. 5. O-antigen production protects E. coli from mechanical shearing
68x58mm (300 x 300 DPI)



172x137mm (300 x 300 DPI)