

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

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

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22

23 **Abstract**

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

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42

43 Introduction

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

51 *E. coli* K-12 is considered to be the archetypal *E. coli* isolate and the premier model organism.
52 Since its isolation in 1922, *E. coli* K-12 has become the workhorse of molecular biology.
53 During this time it has been repeatedly passaged and has been subjected to ionising radiation,
54 ultraviolet light and mutagens, resulting in a number of genetic lesions and an organism which
55 has lost the F plasmid, bacteriophage λ and the ability to produce many surface-associated
56 structures (Hobman *et al.*, 2007, Bachmann, 2004). Indeed, all strains of *E. coli* K-12
57 characterised to date are phenotypically rough, being unable to synthesize O antigen, due to
58 mutations within the *rfb* locus (Fig. 1). In most *E. coli* K-12 strains this is due to the disruption
59 of the *wbbL* gene by an IS5 element, termed the *rfb-50* mutation. By complementing this lesion
60 with plasmid vectors, Reeves and colleagues demonstrated that *wbbL* encoded a rhamnose
61 transferase and *E. coli* K-12 was capable of synthesising O16 serotype LPS (Stevenson *et al.*,
62 1994, Liu & Reeves, 1994).

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

76 Results

77 **Regeneration of the O antigen biosynthesis cluster of *E. coli* K-12.** The *E. coli* K-12 strain
78 MG1655 was the first *E. coli* strain to be sequenced and inspection of its *rfb* locus indicates
79 that it contains an IS5 insertion within *wbbL* (Blattner *et al.*, 1997) (Fig. 1). To confirm that
80 other strain-specific mutations are not responsible for the inability of *E. coli* MG1655 to
81 produce O antigen, we complemented this mutation by cloning an intact version of *wbbL* into
82 the pET20b vector. Fig. 1B shows a silver stained SDS PAGE gel of total cellular protein and
83 LPS and demonstrates that expression of the O antigen is restored in MG1655 by plasmid
84 pET20b/*wbbL* but not by the empty vector or when *wbbL* was inactivated by a single base pair
85 deletion (*i.e.* pET20b/*wbbL*Δ1). Thus, the inability of MG1655 to produce O antigen is due
86 solely to the disruption of *wbbL*.

87 As plasmid complementation can lead to off-target phenotypes due to loss of plasmid
88 maintenance, increased metabolic burdens or the presence of antibiotics to maintain the
89 plasmid, we sought to regenerate the *rfb* cluster on the chromosome. This was achieved using
90 the suicide vector pJP5603/*wbbL*, which was transferred to MG1655 by conjugation. As
91 pJP5603/*wbbL* cannot stably replicate in MG1655, transconjugants can only arise by the
92 integration of the plasmid into the chromosomal copy of *wbbL*. Integration of the plasmid
93 upstream of the IS5 element regenerates the *rfb* cluster, whilst integration downstream does not
94 (Fig. 1C). This led to the generation of two strains, *E. coli* DFB1655 L9, which carries an
95 intact *rfb* cluster, and *E. coli* DFB1655 L5 which carries *wbbL* but maintains the original IS5
96 disruption within *wbbL*. Silver staining for LPS confirmed that both strains synthesized O
97 antigen (Fig. 1D), however, DFB1655 L9 produced considerably more O antigen containing
98 LPS than DFB1655 L5.

99 Research from other groups has shown that O antigen production leads to increased resistance
100 to certain bacteriophages and a decreased ability to be transformed by recombinant plasmids
101 (Ho & Waldor, 2007, Rubires *et al.*, 1997, Stern *et al.*, 1999). Our data show that DFB1655 L9
102 is resistant to phage P1 in phage cross-streak experiments (Fig. S1A) and has decreased
103 transformation efficiency (Fig. S1B) when compared with the strains MG1655 and DFB1655
104 L5. The O antigen protects cells from the bactericidal action of complement (Rubires *et al.*,
105 1997, Grossman *et al.*, 1987). To confirm that the restoration of O antigen biosynthesis affects
106 the ability of MG1655 to survive complement-mediated killing, we exposed our strains to
107 undiluted human serum and assessed their viability over 3 h. Data in Fig. S1C shows that all
108 MG1655 and DFB1655 L5 cells were killed within 45 minutes but that DFB1655 L9 was

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

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

134 The integrity of the outer membrane was assessed by a variety of techniques. First, Western
135 blotting determined that the levels of the major outer membrane porins OmpF, OmpC and
136 OmpA and the essential outer membrane lipoprotein BamD (Fig. S3A) were indistinguishable
137 when DFB1655 L9 was compared to MG1655. Next, we examined the accumulation of the dye
138 Hoechst 33342, a substrate of the major AcrAB-TolC efflux system in the presence and
139 absence of the efflux pump inhibitor phenyl-arginine- β -naphthylamide (PA β N) (Coldham *et al.*,
140 2010, Webber *et al.*, 2008). Data in Fig. S3B shows that accumulation of Hoechst 33342
141 was similar in all strains, indicating chemical efflux was unaltered by the presence of the O

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

147 **O antigen production enhances killing of *C. elegans*.** Previous experiments have
148 demonstrated that *E. coli* K-12 is unable to colonise the intestinal tract of humans and animals.
149 Furthermore, *E. coli* K-12 is often used as a negative control in models of pathogenicity. We
150 wished to determine if restoration of the O antigen had an impact on the ability of *E. coli* to
151 colonise the intestinal tract and/or mediate disease. The hermaphroditic worm *C. elegans* is
152 often used as a model for microbial pathogenicity. Therefore, we investigated the ability of
153 each of our strains to colonise and kill *C. elegans* using a slow kill assay, in which nematodes
154 are fed bacteria and their viability monitored over 10 days (Aballay *et al.*, 2003). Results in
155 Fig. 2A show that DFB1655 L9 killed *C. elegans* more rapidly than MG1655, DFB1655 L5
156 and the normal food source *E. coli* OP50 (Brenner, 1974). Importantly, DFB1655 L9 killed *C.*
157 *elegans* at a rate almost identical to the pathogenic strain *Pseudomonas aeruginosa* PA14 (Fig.
158 2A). Previous investigations have demonstrated that the growth medium may alter the rate at
159 which *C. elegans* succumbs to bacterial infection. To investigate if this was true for *E. coli* K-
160 12 strains we repeated the above experiments on BHI agar plates. As reported previously,
161 when grown on BHI agar *C. elegans* becomes more susceptible to the normal food source *E.*
162 *coli* OP50 (Garsin *et al.*, 2001). Whilst MG1655 and DFB1655 L5 are more pathogenic for *C.*
163 *elegans* when grown on BHI plates, importantly, DFB1655 L9 kills *C. elegans* significantly
164 faster than the these strains ($p < 0.05$) (Fig. 2B). These data indicate that O antigen production
165 increases the virulence of the laboratory strain MG1655 and that despite decades of laboratory
166 growth it has retained the ability to kill *C. elegans*.

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

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

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

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

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

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

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

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

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

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

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

257 Discussion

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

273 We found the rough strain MG1655 was more genetically tractable; O antigen-expressing
274 DFB1655 L9 was more resistant to bacteriophage P1 and to transformation with plasmids.
275 These phenotypes can be simply explained by the O antigen acting as a barrier; as the P1
276 receptor is the LPS core it is likely that O antigen obscures this region of LPS and prevents
277 phage attachment and infection (Ho & Waldor, 2007). It has been suggested that loss of O
278 antigen production was a result of *E. coli* K-12 adaptation to laboratory life (Hobman et al.,
279 2007). Indeed, loss of O antigen production by a similar IS-inactivation mechanism is a
280 common occurrence for laboratory strains since the *rfb* clusters of both the *E. coli* B and C
281 laboratory strains, which have different evolutionary lineages to *E. coli* K-12, are also
282 disrupted by IS elements (Jeong *et al.*, 2009, Michel *et al.*, 2010, Crossman *et al.*, 2010). Thus,
283 it is tempting to speculate that the reason that rough *E. coli* laboratory strains predominate is
284 due to selection by early microbiologists because of their ease of manipulation.

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

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

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

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

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

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

353 In conclusion, two major findings can be derived from this study. First, the O antigen density is
354 critically important for a variety of phenotypes and when creating genetic mutations
355 researchers must be cognisant of the fact that perturbations in O antigen density can have a
356 major impact on phenotype. Secondly, as *E. coli* K-12 and HS are not considered to be
357 pathogenic organisms, our results directly question whether *C. elegans* is a suitable model for
358 studying the virulence of pathogenic *E. coli*. Finally, since its isolation in 1922 *E. coli* K-12
359 has also lost the ability to produce O antigen, capsule, has been cured of the F plasmid and
360 phage lambda, and has accumulated numerous other deleterious mutations (Hobman *et al.*,
361 2007, Bachmann, 2004, Peleg *et al.*, 2005). These alterations have had a profound impact on *E.*
362 *coli* K-12 biology and suggest that our heavy reliance on *E. coli* K-12 as a model organism
363 may be ill-founded.

364 **Experimental Procedures**

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

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

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

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

401 **Nematode virulence assays.** *C. elegans* strain Bristol N2 was cultured with *E. coli* strain
402 OP50 using standard methods and survival assays were performed as before (Aballay et al.,
403 2003). Larval stage 4 *C. elegans* were picked and transferred onto assay plates, with 20 worms
404 on each plate. Plates were incubated at 25°C and scored daily for survival. Worms were

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

411 **Phenotypic analyses.** To test for phage P1 resistance, strains were cross-streaked against a P1
412 lysate as before (Ho & Waldor, 2007). Biofilm formation was examined on polystyrene
413 surfaces using 96-well microtitre plates as before (Raghunathan *et al.*, 2011). Flow chamber
414 experiments were performed as before (Wells *et al.*, 2008) and biofilms were formed on glass
415 surfaces in a multichannel flow system. Strains were transformed with the GFP-expressing
416 plasmid pJB42. Biofilm development was monitored using a confocal scanning laser
417 microscopy. Appropriate z-stacks were collected for each strain and analyzed by using the
418 COMSTAT software program (Heydorn *et al.*, 2000). Biofilm experiments were performed in
419 triplicate. Serum killing assays were performed as before (Raghunathan *et al.*, 2011) with
420 viable counts determined after 45, 90 and 180 min. For EGTA experiments, EGTA and MgSO₄
421 was added to the serum at 10mM and 5mM concentrations respectively before incubating with
422 bacteria. Experiments were performed in triplicate. Phenotype microarray growth was
423 performed by Biolog Inc. (Hayward, California USA) as described previously (Bochner *et al.*,
424 2001, Zhou *et al.*, 2003). Sensitivity to triclosan was investigated by examining the growth of
425 strains in liquid culture. The optical density was monitored using a FluoStar Optima (BMG
426 labtech, U.K). For each strain two biological replicate overnight cultures were used to inoculate
427 four technical replicate cultures each and 200 µl of each culture added to separate wells of a
428 microtitre tray which was then incubated at 37°C with shaking (Andrews, 2001). Triclosan was
429 injected after 90 min incubation. Membrane permeability and efflux activity were examined by
430 measuring the accumulation of Hoechst 33342. Experiments were performed in the presence
431 and absence of the efflux pump PAβN as previously described (Coldham *et al.*, 2010, Webber
432 & Coldham, 2010). Mechanical shearing was performed by incubating concentrated cultures
433 (OD₆₀₀:10) with or without 12 glass beads. Tubes were shaken at 400 rpm for 12 h. Samples
434 were taken from the tubes at 12 h for dilution and plating on LB agar. Following overnight
435 CFUs were calculated and the difference between 0 and 12 h expressed as Log₁₀ of CFU ml⁻¹.
436 Experiments were performed in triplicate on two separate occasions. Lysozyme resistance was
437 examined by growing strains to OD₆₀₀:0.6 in LB broth. Either 0, 1 or 2 mg ml⁻¹ of lysozyme
438 (Sigma) was added to the cultures and shaken at 37°C for 2 h before dilution and plating on LB

439 agar. After incubation CFU ml⁻¹ were calculated. 1 mg ml⁻¹ lysozyme with 10 mM EDTA was
440 also added to cultures as a control. Experiments were performed in triplicate on three separate
441 occasions.

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601
602

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

604 A. Organisation of the *rfb* cluster from *E. coli* K-12 strain MG1655, showing the IS5
605 inactivation of *wbbL* (*i.e.* the *rfb-50* mutation) and the approximate deletion of the *rfb* cluster in
606 *E. coli* K-12 strain WG1 (*i.e.* the *rfb-51* mutation).

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

611 C. Integration of the conjugative suicide vector pJP5603/ *wbbL* into the chromosome of
612 MG1655. Integration upstream of the IS5 element (recombination event 1) regenerates a
613 complete *rfb* cluster (strain DFB1655 L9), whilst integration downstream of the IS5 element
614 (recombination event 2) does not (strain DFB1655 L5). Note that *gnd* is not part of the *rfb*
615 cluster and thus the insertion of pJP5603/ *wbbL* will not cause polar effects on its expression.
616 Silver stained SDS PAGE gels of total protein and LPS samples from *E. coli* K-12 strains.

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

621

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

623 A. Nematode slow-kill virulence assays on NGM agar. The ability of each strain to kill the
624 nematode worm *C. elegans* was examined over a 10-day period. The LT₅₀ (time for half of the
625 worms to die) was calculated for each experiment: *E. coli* MG1655 LT₅₀=9.36; DFB1655 L5
626 LT₅₀=7.318 and DFB1655 L9 LT₅₀=4.52. The *E. coli* strain OP50, a commonly used as a food
627 source for *C. elegans*, was used as a negative control (LT₅₀>10). The opportunistic pathogen
628 *Pseudomonas aeruginosa* PA14 (LT₅₀=3.54), which was previously shown to kill *C. elegans*,
629 was included as a positive control.

630 B. Killing of *C. elegans* was monitored during growth of MG1655 (LT₅₀=6.54), DFB1655 L5
631 (LT₅₀=6.42) and DFB1655 L9 (LT₅₀=4.72) on BHI agar. *E. coli* OP50 was included as a
632 negative control (LT₅₀>10).

633 C. Nematodes were fed *E. coli* 042 (LT₅₀=4.09), DFB042, a mutant strain lacking O-antigen
634 expression (LT₅₀=5.90), the commensal strain *E. coli* HS (LT₅₀=4.81) or MG1655 (LT₅₀=9.82).
635 *C. elegans* killing was assessed. The effect of LPS on nematode survival was examined by
636 feeding worms MG1655 supplemented with purified O-antigen containing LPS (LT₅₀=10).

637

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

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

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

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

649

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

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

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

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

657

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

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

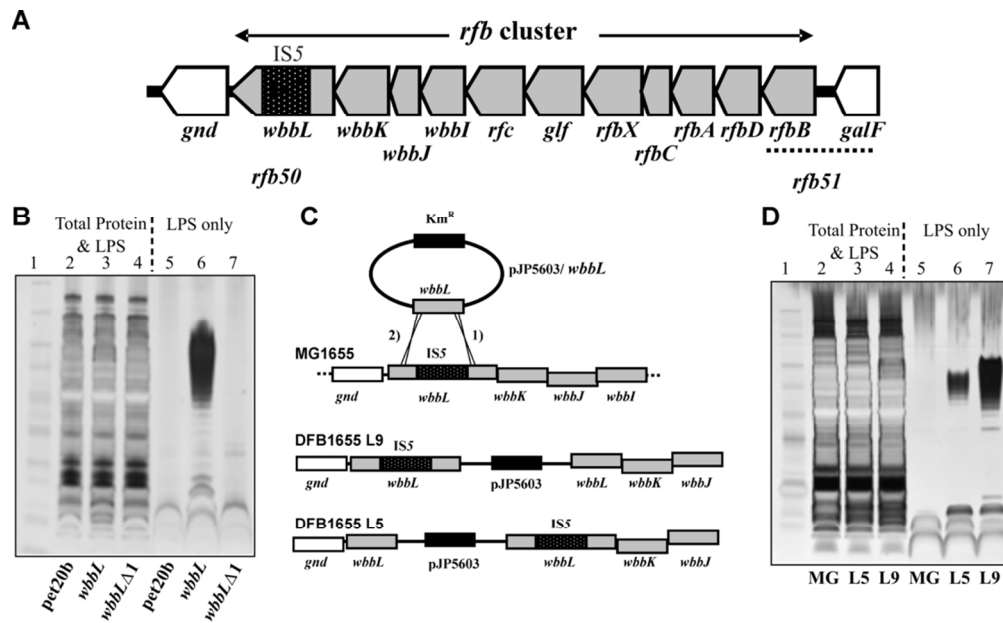


Fig. 1. Regeneration of the *E. coli* K-12 *rfb* O antigen biosynthetic locus
98x60mm (300 x 300 DPI)

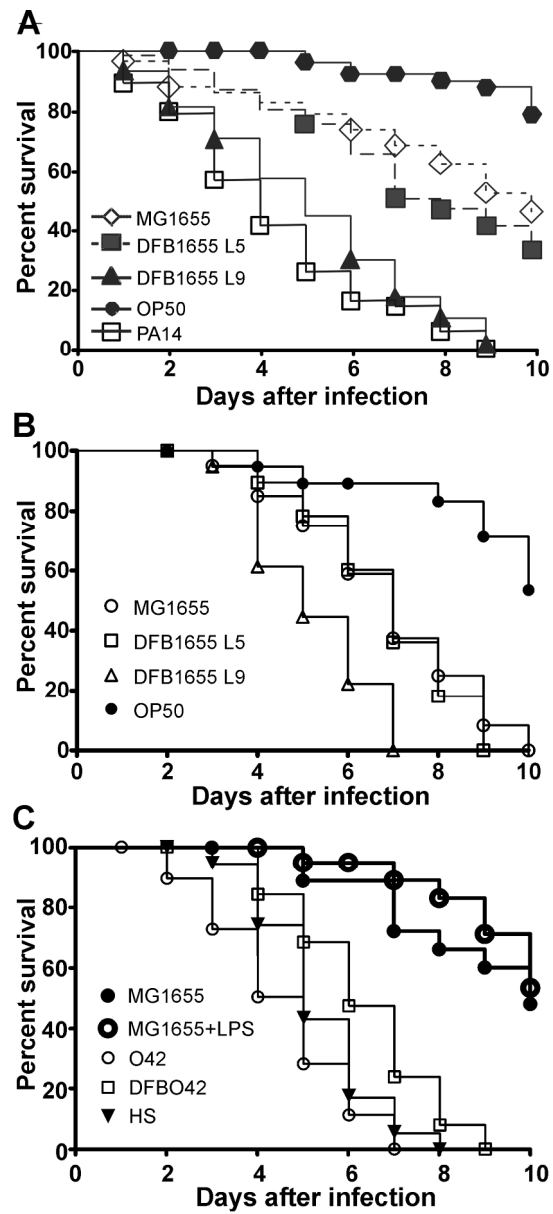


Fig. 2. O-antigen biosynthesis enables *E. coli* K-12 to kill *C. elegans*
152x340mm (300 x 300 DPI)

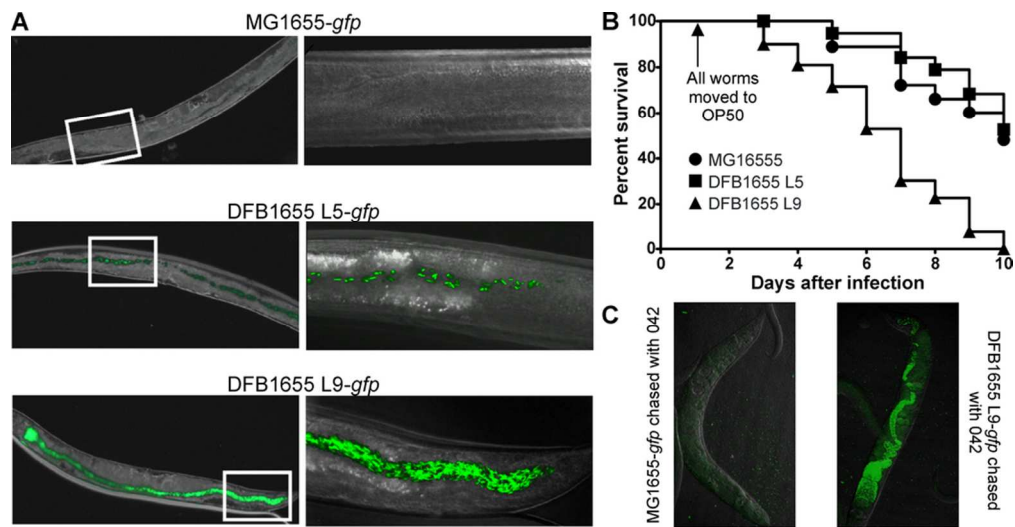


Fig. 3. DFB1655 L9 colonises *C. elegans* intestine
88x46mm (300 x 300 DPI)

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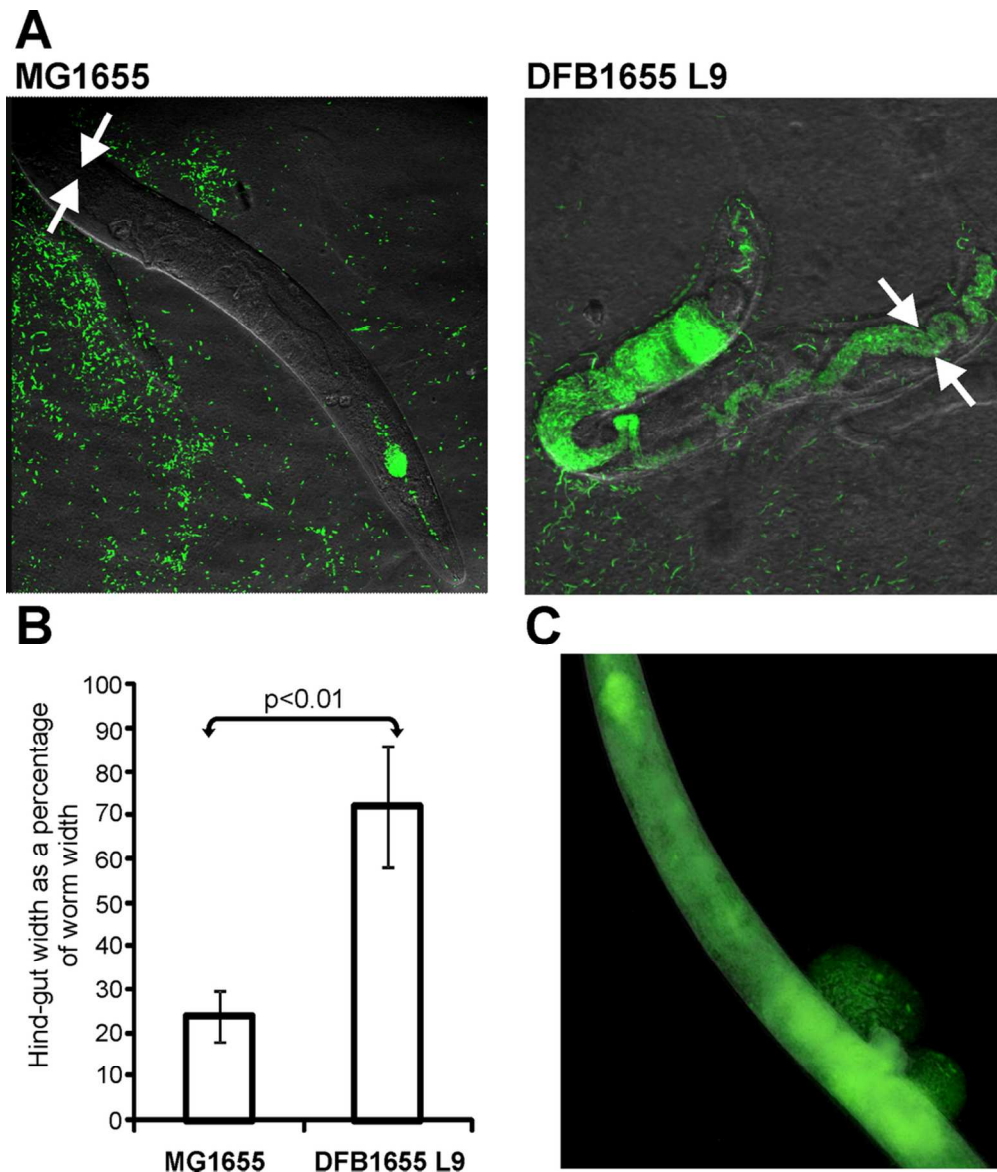


Fig. 4. Colonisation of *C. elegans* by DFB1655 L9 leads to distension of the nematode gut.
92x107mm (300 x 300 DPI)

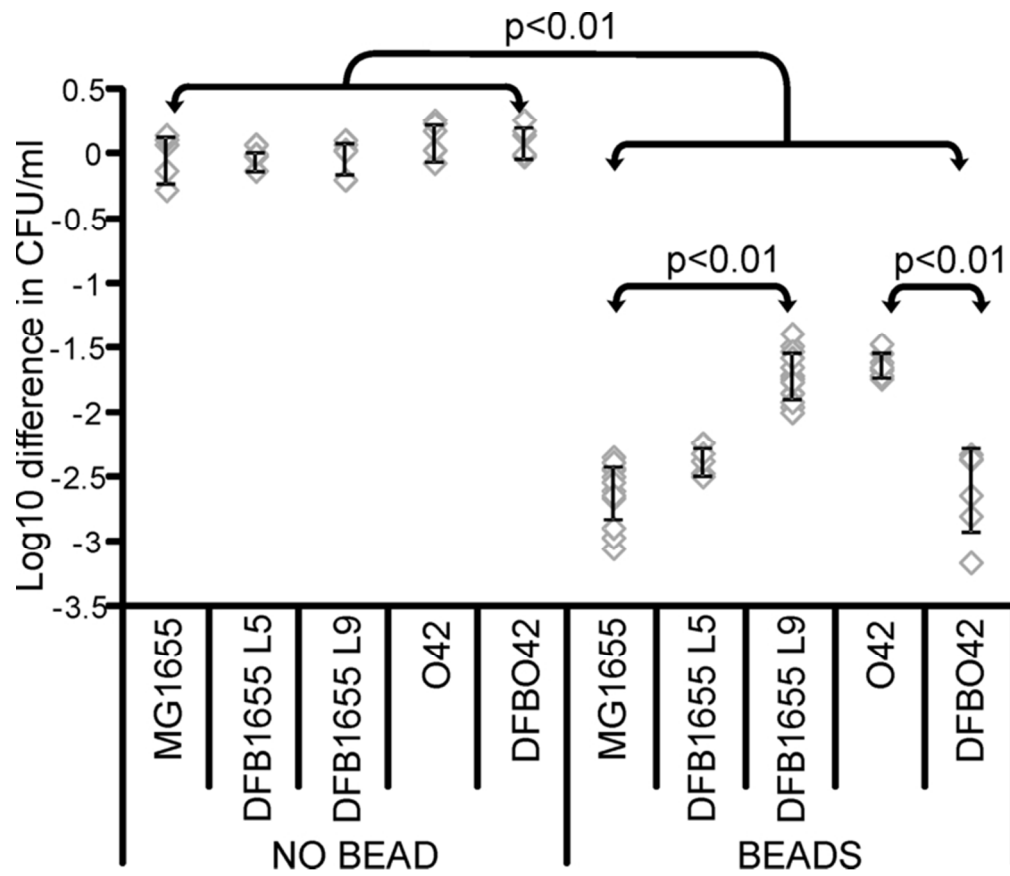
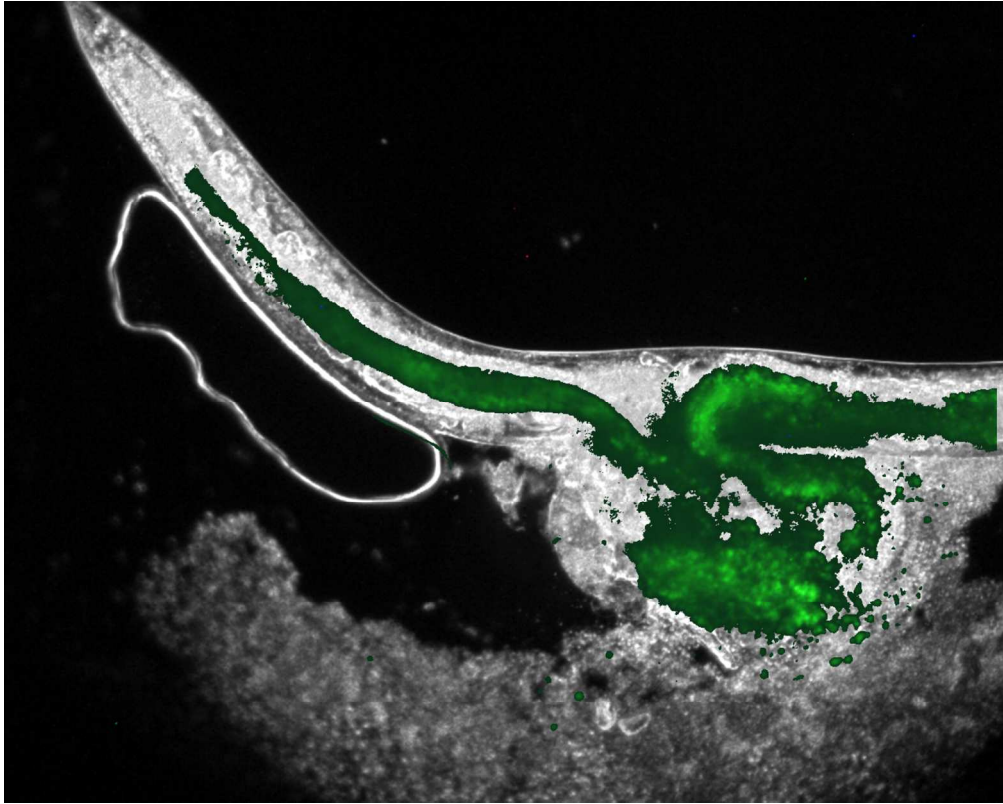


Fig. 5. O-antigen production protects E. coli from mechanical shearing
68x58mm (300 x 300 DPI)



172x137mm (300 x 300 DPI)

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