Research article

Motility is required for the competitive fitness of entomopathogenic
Photorhabdus luminescens during insect infection

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Abstract

Background: Photorhabdus are motile members of the family Enterobactericeae that are pathogenic to insect larvae whilst also maintaining a mutualistic interaction with entomophagous nematodes of the family Heterorhabditidae. The interactions between Photorhabdus and its hosts are thought to be an obligate part of the bacteria’s life-cycle in the environment. Motility often plays a key role in mediating bacteria-host interactions and, in this study, we were interested in characterising the role of motility in the Photorhabdus-nematode-insect tripartite association.

Results: We constructed deletion mutants of flgG (blocking flagella production) and motAB (blocking flagella rotation) in P. luminescens TT01. Using these mutants we show that both the ΔflgG and ΔmotAB mutants are equally as good as the wild-type (WT) bacteria in killing insects and supporting nematode growth and development suggesting that flagella production and motility are not required for pathogenicity or mutualism. However we show that the production of flagella is associated with a significant metabolic cost during growth on agar plates suggesting that, although not required for pathogenicity or mutualism, there must be a strong selective pressure to retain flagella production (and motility) during the interactions between Photorhabdus and its different hosts. To this end we show that both the ΔflgG and ΔmotAB mutants are out-competed by WT Photorhabdus during prolonged incubation in the insect revealing that motile bacteria do have a fitness advantage during colonisation of the insect larva.

Conclusion: This is the first report of a role for motility in Photorhabdus and we show that, although not required for either pathogenicity or mutualism, motility does contribute to the competitive fitness of Photorhabdus during infection of the insect (and, to a lesser extent, the nematode). This adaptive function is similar to the role ascribed to motility in mammalian pathogens such as uropathogenic Escherichia coli (UPEC). Therefore, in addition to describing a role for motility in Photorhabdus, this study reinforces the relevance and utility of this bacterium as a model for studying bacteria-host interactions.

Background

Photorhabdus are Gram negative bacteria that are highly virulent pathogens of a wide variety of insect larvae whilst also maintaining a mutualistic interaction with nematodes from the family Heterorhabditidae. Photorhabdus normally colonise the intestinal tract of the infective stage...
of the nematode, the infective juvenile (IJ). The IJs are a
non-feeding stage of the nematode that lives in the soil
where they actively seek out potential larval hosts. On
finding a suitable host the IJ enters the larva and the bac-
teria are regurgitated into the insect hemolymph. Phy-
torhabdus actively circumvent the insect innate immune
system by inhibiting and adapting to the humoral
response whilst also suppressing phagocytosis by circulat-
ing haemocytes [1-3]. During infection of the insect the
bacteria grow exponentially, producing a wide range of
toxins and hydrolytic exoenzymes that are responsible for
the death and subsequent bioconversion of the insect
larva into a nutrient soup that is ideal for nematode
growth and development [4,5]. The nematodes feed on
the bacterial biomass within the insect cadaver and
develop through juvenile (J1–J4) stages to form adult
males and females. After several rounds of reproduction
the J1 stage nematodes receive uncharacterised environ-
mental cues that stimulate the development of IJs. At this
point the developing IJs are colonised by Photorhabdus
before they emerge from the insect cadaver to find new
hosts (for recent reviews see [6,7]).

Many bacteria are motile through the action of large com-
plex protein assemblages called flagella. The production
and function of flagella are best studied in the enteric bac-
teria Escherichia coli and Salmonella enterica serovar Typh-
imurium where it has been shown that the expression of
genes required for flagella-mediated motility (and chem-
otaxis) is controlled by a complex regulatory network
[8,9]. Flagellum-mediated motility often plays a key role
in mediating different bacteria-host interactions. For
example motility is important during the colonisation of
the squid by Vibrio fischeri and also during the infection of
mammals by both Salmonella and E. coli [10-14]. Pho-
torhabdus are motile through the action of peritrichously
arranged flagella and we hypothesised that motility must
play some role in the interactions between Photorhabdus
and its invertebrate hosts. This was based on the principle
that unused or redundant traits in bacteria will be lost
over time [15]. Therefore we constructed specific flagel-
llum-minus (Fla-) and non-motile (Mot-) mutants of Phy-
torhabdus and, using these mutants, we show that motility
is not required for either pathogenicity or mutualism.
However we do show that WT bacteria out-compete non-
motile mutants during prolonged incubation in insect
cadavers suggesting that motility confers a fitness advan-
tage during colonisation of the insect.

Results

Construction of mutations in flgG and motAB

The genome sequence of P. luminescens TT01 is available
and, by comparison with the closely related genome of E.
coli, the TT01 genome is predicted to contain 49 genes
required for the production and assembly of functional
flagella and chemotaxis [16]. The motility-associated
genes are found as 4 distinct genetic loci on a large
(approx. 130 Kb) fragment of TT01 genomic DNA stretch-
ing from nucleotide 2195317 to 2322562 (Fig. 1). To
examine the role of motility in P. luminescens TT01 we
constructed mutations in genes that are known to be
required for motility in other bacteria, flgG and motAB.
The flgG gene encodes the distal rod protein of the flagel-
lar hook-basal body (HBB) and mutations in flgG would
be expected to prevent completion of this structure and,
therefore, flagella assembly (Fla-). The motAB genes
encode the motor-force generator that is required for rota-
tion of the flagella and strains carrying mutations in
motAB can assemble normal flagella but the flagella
cannot rotate (Mot-). Using a strategy that results in the con-
struction of unmarked, non-polar deletions we
completely removed the flgG gene such that only the start
and stop codons remained. However the motAB operon
overlaps with the downstream cheA gene and, to prevent
any polar affects on cheA expression, the last 20 nucle-
otides of the motB gene (containing the predicted ribos-
ome-binding site for the cheA gene) were not deleted. We
confirmed that BMM800 (ΔflgG) and BMM802 (ΔmotAB)
were non-motile using swim agar and that motility could
be restored by the in trans expression of the respective
genes from a plasmid i.e. pBMM800 (flgG) and pBMM802
(motAB) respectively (Fig. 2).

Motility is required for attachment to surfaces

In E. coli (and other bacteria) it has been shown that flag-
ella are important for bacterial attachment to abiotic sur-
faces [17-20]. Therefore the wells of a polypropylene
microtitre plate were inoculated with TT01, BMM800
(ΔflgG) and BMM802 (ΔmotAB) and the plate was incu-
bated at 30°C without shaking for 24 h, 48 h and 72 h.
Bacterial attachment was quantified using crystal violet
and it is clear that both the ΔflgG and ΔmotAB mutants
were severely affected (5–10-fold) in their ability to attach
to the walls of the microtitre plates when compared to the
WT bacteria (Fig. 3). Attachment was restored to the
mutants carrying a plasmid expressing the appropriate
gene(s). Therefore, as in other enteric bacteria, motility is
required for attachment of Photorhabdus to abiotic sur-
faces.

There is a cost associated with the production of flagella

Although flagella are generally not required for growth
their production and assembly can be costly to the cell in
terms of the utilisation of resources [21]. To determine
whether the production of flagella is costly to Photorhab-
dus we set up a competition experiment whereby lipid
agar plates were inoculated with 50:50 mixtures of WT
bacteria with either the ΔflgG or the ΔmotAB mutant. The
plates were incubated at 30°C and the relative abundance
of each bacterial strain on the agar plate was measured at
time intervals post-inoculation i.e. 3 days and 21 days. In preliminary tests using LB broth we established that the growth rates of the ΔflgG and the ΔmotAB mutants were identical to WT (data not shown). However, when grown for an extended period of time on lipid agar plates, we observed that the ΔflgG mutant was present at higher levels than the WT (a ratio of 85:15 on day 21) on the plate (Fig. 4A). On the other hand the ΔmotAB mutant maintained a 50:50 ratio with the WT throughout the 21 days (Fig. 4B). Therefore there is a measurable cost associated with the production of flagella, but not motility per se, during Photorhabdus growth in vitro.

Motility is required for the competitive fitness of Photorhabdus during insect colonization

To test for a possible role during insect virulence, we injected 200 cfu of TT01, BMM800 (ΔflgG) or BMM802 (ΔmotAB) into final instar Galleria mellonella larvae. The LT$_{50}$ (i.e. time taken to kill 50% of the insect larvae) values of the WT and mutant bacteria were similar showing that motility is not required for Photorhabdus virulence against insects (data not shown). To test whether motility might confer an advantage at some point during infection of the insect we injected a 50:50 mixture of TT01 with either the ΔflgG or the ΔmotAB mutant into G. mellonella larvae and incubated the larvae at 25°C for either 3 or 21 days (all insects were dead after 2 days). The insects were then homogenised and the proportion of motile to non-motile bacteria was determined using swim agar, as described. After 3 days in the insect the ΔflgG mutant was present at slightly higher levels than the WT but this trend was reversed after 21 days at which time the WT predominated in the insect cadaver (Fig. 5A). Similarly the WT had almost completely out-competed the ΔmotAB mutant.

Figure 1

The genetic loci encoding the genes required for flagella production and motility in P. luminescens TT01. By comparison with the E. coli and Salmonella genomes (available at ColiBase http://coli.base.bham.ac.uk) we identified 4 genetic loci (labelled I-IV) predicted to be involved in the production of flagella and chemotaxis in Photorhabdus. The numbers shown at beginning and end of each locus represents the genetic location on the Photorhabdus genome (according to PhotoList http://genolist.pasteur.fr/PhotoList). The genes deleted in this study (motAB and flgG) are indicated in grey.
after 21 days in the insect (Fig. 5B). Therefore motility confers a fitness advantage to *Photorhabdus* during the prolonged incubation in the insect normally experienced by these bacteria as part of their life-cycle. This would suggest that the cost associated with the production of flagella is offset by the ability of *Photorhabdus* to be motile in the insect.

Motility affects the ability of *Photorhabdus* to colonise the nematode
To test for a role for motility during the interaction with *Photurhabdus* we inoculated lipid agar plates with overnight cultures of TT01, BMM800 (ΔflgG) or BMM802 (ΔmotAB). After 3 days at 25°C the bacterial biomass on the plates was seeded with 50 surface-sterilised IJ nematodes and incubated, in the dark, at 25°C for a total of 21 days. During this time 3 aspects of the bacteria-nematode interaction are routinely monitored: 1) the fraction of inoculated IJs that exit diapause to develop into adult hermaphrodites (i.e. this is called IJ recovery and it is an indicator of the ability of the bacteria to produce the signals required to stimulate IJ recovery); 2) the total number of new generation IJs that are recovered after 21 days (i.e. this is called the IJ yield and it is an indicator of the ability of the bacteria to support nematode growth and reproduction) and 3) IJ colonisation by the bacteria (required for the continuation of the mutualism between bacteria and nematode). We did not observe any defect in either IJ recovery or IJ yield suggesting that bacterial motility does not make a significant contribution to nematode growth and development (data not shown). On the other hand we did observe that the ΔflgG mutant was present in the IJ at levels significantly higher than the WT (median for ΔflgG = 159 cfu/IJ compared to median for WT = 105 cfu/IJ; *P* = 0.0478) whilst, in contrast, the ΔmotAB mutant was present in the IJ at significantly lower levels than the WT (median for ΔmotAB = 82 cfu/IJ compared to median for WT = 105 cfu/IJ; *P* = 0.0065) (Fig. 6). Therefore, although not required for colonisation, the ability to produce flagella and motility do appear to have contrasting affects on the final number of bacteria present in the IJ.

Discussion
*Photurhabdus* are highly virulent to a wide range of insect larvae and, following insect death the bacteria must remain in the insect cadaver at high densities for extended periods of time (up to 2–3 weeks under optimal conditions) to facilitate nematode growth and development. We have shown that, although neither flagella production nor motility is required for pathogenicity, there is a significant advantage to being motile during the normally prolonged incubation in the insect.

Figure 2
Swimming motility of *P. luminescens*. The WT was transformed using the plasmid pTRC99a (TT01 + vector) and the mutants BMM800 (ΔflgG) and BMM802 (ΔmotAB) were transformed using pTRC99a (BMM800 + vector and BMM802 + vector) or either pBMM800 (BMM800 + pBMM800) or pBMM802 (BMM802 + pBMM802). Cells were grown overnight at 30°C in LB broth (+ Amp) and diluted to an OD600 = 1.0 in fresh LB before 5 μl was spotted onto the surface of a swim agar plate. Plates were incubated at 30°C for 44 h before motility was scored.
Our data show that, on agar plates, the ΔmotAB mutant is as competitive as the WT suggesting that there is no perceived benefit to being motile in this environment. This was not unexpected as the concentration of agar used would prevent cells from swimming or swarming, whether they are motile or not, thus rendering the ΔmotAB mutant neutral in this environment. On the other hand, the ΔflgG mutant has a considerable advantage over WT suggesting that there is a metabolic cost associated with the production of flagella. In a recent study Fontaine et al. (2008) showed that the reduced mortality associated with a fliA mutation in E. coli was probably due to decreased internal cell stress due to the absence of physical destabilisation of the membrane [21]. Therefore the cost associated with flagella production in Photorhabdus may be due to the utilisation of resources and energy for the production and/or function of the flagella or it may be due to the stresses associated with assembly of the flagella through the cellular membrane. In the insect this trend is reversed and the ΔflgG cells are now disadvantaged during extended growth (i.e. 21 days). This would suggest that flagella production benefits the bacteria during insect infection. Therefore the costs of flagella production (and presumably motility) appear to be offset during extended incubation in the insect. In support of this, the ΔmotAB mutant, that still bears the cost of producing flagella and yet does not derive any benefit associated with motility in the insect, is present at much lower levels than the ΔflgG mutant in competition assays after 21 days.

The frequency of motile cells observed throughout the growth of Photorhabdus under normal culturing conditions (i.e. shaking at 30°C in LB broth) is very low (<< 1%) (our unpublished data). During growth under static conditions the frequency of motility in the population increased reaching a maximum of 30% at 16 h post-inoculation before rapidly decreasing (data not shown). Therefore motility is restricted, both spatially and temporally, during the growth of Photorhabdus. This limited exposure of motility to any selection pressure in the environment could explain the relatively modest differences in fitness observed in this study.

In uropathogenic E. coli (UPEC) motility has been shown to contribute to the fitness of the bacteria during colonisation of the urinary tract [22]. Indeed recent studies have shown that motility facilitates the movement of UPEC to the upper urinary tract [12]. Moreover motility has been shown to be required for Salmonella to access high-energy nutrients found at sites of inflammation in the mamma-
lian gut [13]. In the same way we expect that motility in *Photorhabdus*, a closely related bacterium, will facilitate the movement of the bacteria throughout the insect cadaver thus enabling invasion of nutrient-rich niches that facilitate growth of the motile strain. In this regard *Photorhabdus* would also be expected to undergo chemotaxis and the genome is predicted to encode a complete Che signaling system in addition to 2 methyl-accepting chemotaxis proteins i.e. MCPs (see Fig. 1). However the role of chemotaxis was not investigated in this study.

We have shown that neither flagella production nor motility is required for the mutualistic association between *Photorhabdus* and the *Heterorhabditis* nematode.

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**Figure 4**

**Competition assays on agar plates.** Equal numbers of A) TT01 and BMM800 (Δ*flgG*) and B) TT01 and BMM802 (Δ*motAB*) were mixed and inoculated onto each of 5 lipid agar plates. The proportion (%) of motile:non-motile bacteria was determined in the initial mixture before inoculation and this is reported as Day 0. At the other time points the proportion of motile:non-motile bacteria was determined on each of the 5 individual plates. The results shown are the mean ± SD of 3 experiments.
Photorhabdus has 2 roles during the interaction with the nematode: 1) the bacteria must provide nutrients for the nematode and 2) the bacteria must colonise the IJ. The nematodes feed on the Photorhabdus biomass that is present either in the insect or on agar plates and, therefore, the nematodes obtain a substantial part of their required nutrients directly from the bacteria. We did not see any differences in the growth and development of the nematodes growing on agar plates with the WT bacteria compared to the ΔflgG and ΔmotAB mutants. Indeed infecting insects with IJs colonised with either ΔflgG or ΔmotAB mutants resulted in an IJ yield (a quantitative indicator of the ability of the bacteria to support nematode growth and development) very similar to that observed with IJs colonised with WT bacteria (data not shown). Therefore flagella production and motility do not appear to play any role in the nutritional interaction.

Figure 5

**Competition assays in the insect.** Equal numbers of A) TT01 and BMM800 (ΔflgG) and B) TT01 and BMM802 (ΔmotAB) were mixed and injected into G. mellonella larvae. The proportion (%) of motile:non-motile bacteria was determined in the initial mixture before injection and this is reported as Day 0. At Day 3 and Day 21 the proportion of motile:non-motile bacteria was determined in each of 10 individual insects. The results shown are the mean ± SD of 3 experiments.
between Photorhabdus and the nematodes either on agar plates or in the insect.

Photorhabdus are maternally transmitted from the hermaphrodite stage nematode (i.e. the mother) to the developing IJ [23]. As the Heterorhabditis nematodes feed on Photorhabdus some viable bacteria enter the lumen of the gut of the hermaphrodite and attach to specific cells in the distal region of the gut (specifically the INT9 cells). The bacteria infect the neighbouring rectal glands cells and replicate within vacuoles. The rectal gland cells rupture, releasing Photorhabdus into the body cavity of the hermaphrodite where the bacteria encounter and colonise the developing IJs [23]. The IJ is initially colonised by 1–2 bacteria that subsequently replicate, resulting in a final population of approximately 100 cfu of WT bacteria per IJ. The proportion of IJs colonised by the ΔflgG and ΔmotAB mutants is the same as the WT (i.e. approx. 80% in all cases) suggesting that attachment to the IJ, and presumably infection of the hermaphrodite, is independent of flagella and/or motility. On the other hand, the final population level of Photorhabdus within the IJ is significantly altered in nematodes that are cultured on either the ΔflgG or the ΔmotAB mutant strain. Therefore IJs that have been grown on the ΔflgG mutant carry, on average, a bacterial population that is 50% greater than the population within IJs cultured on WT bacteria. In contrast the ΔmotAB mutant does not reach population levels within the IJ that are equivalent to the WT suggesting that the production of non-functional flagella negatively influences growth in the nematode. These results might be explained in terms of the metabolic cost associated with the production of flagella. Therefore, in the absence of flagella production, the ΔflgG mutant may be able to put more of the limited resources available within the nematode into biomass production and division. On the other hand the ΔmotAB mutant still bears the cost of producing flagella although these are non-functional. Interestingly the fact that the ΔmotAB mutation is not neutral, in terms of IJ colonisation, suggests that Photorhabdus are likely to be motile at some point during the colonisation of the nematode. The bacteria initially colonise the proximal end of the IJ gut and one possibility is that motility may facilitate exploration of the distal regions of the gut thus allowing the bacteria to make better use of the limited resources available within the nematode.

Conclusion
In this study we show that there is a significant metabolic cost associated with the production of flagella (and motility) in Photorhabdus. Nonetheless Photorhabdus are motile suggesting that motility is an adaptive trait that is under powerful positive selection in the environments where Photorhabdus is normally found i.e. in the insect and nematode. In this study we show that, although motility is not required for either pathogenicity or mutualism, this trait is advantageous during the interactions between Photorhabdus and both of its invertebrate hosts. Therefore, in addition to describing a role for motility in Photorhabdus, this work also highlights the functional overlap between pathogenicity and mutualism and reinforces the utility of Photorhabdus as a model for studying these different bacteria-host interactions.

Methods

Bacterial strains and culture conditions
A spontaneous rifampicin-resistant mutant of Photorhabdus luminescens subsp. luminescens was used as the wild-type (WT) in all experiments [2]. The bacteria were cultured in LB broth or on LB agar (LB broth plus 1.5% (w/v) agar) at 30°C for P. luminescens. Unless otherwise stated all LB agar plates were supplemented with 0.1% (w/v) pyruvate [24]. Escherichia coli S17-1 (λpir) and E. coli
EC100 (Epicentre) were cultured at either 30°C or 37°C as indicated. Swim agar is LB broth plus 0.3% (w/v) added agar. When required antibiotics were added at the following concentrations: ampicillin (Ap), 100 μg ml⁻¹; chloramphenicol (Cm), 20 μg ml⁻¹ and rifampicin (Rif), 50 μg ml⁻¹.

**Construction of deletion mutants**

The *flgG* and *motAB* genes were deleted using a previously described procedure [25]. This method results in the construction of unmarked and non-polar deletion mutation of the selected gene(s). For the deletion of each gene fragment A (approx. 600 bp upstream of the target gene) and fragment B (approx. 600 bp downstream of the target gene) were amplified by PCR using KOD Hi-Fi polymerase (Novagen) and the primer pairs KO1 + KO3 (for fragment A) and KO2 + KO4 (for fragment B). The primer sequences are listed in Table 1. The fragments were purified using the Qiagen PCR clean up kit, combined and subjected to 10 cycles of primerless PCR to allow the polymerase to anneal fragments A and B (as KO3 and KO4 are complimentary). The annealed fragment was used as a template in a final PCR, using the appropriate KO1 and KO2 primers, to enrich for a full-length fragment. The different KO1 and KO2 primers incorporate restriction sites to facilitate cloning into the suicide vector pDS132 [26]. The resulting pDS132-*flgG* and pDS132-*motAB* plasmids were isolated and used to transform *E. coli* S17-1 λpir (the donor) for conjugation into *P. luminescens* TT01 (the recipient). Conjugation and selection of the appropriate exconjugants was carried out as described previously [25]. Deletion mutants were then identified by colony PCR using the KO5 and KO6 primers (a deletion will result in a PCR product of a predictable size). The amplification product of each mutant was sequenced to confirm the integrity of the knock out allele.

**Cloning of the *flgG* and *motAB* genes**

The *flgG* and *motAB* genes were amplified from *P. luminescens* TT01 genomic DNA by PCR using KOD polymerase. The primers used for *flgG* were CAT001 (5'-TAAAACCCATGGTCCGATCATTATGGATTGC-3') and CAT002 (5'-GCTGGATCTAGAATTAACTAGTGCTTTTGAGCTGCTTTAGC-3') and for *motAB* CAT003 (5'-GATATCCCATGGTAGTACTTTTAGGATATATC-3') and CAT004 (5'-GCAGTGTCTAGATTACTTTTGGTGTCACCTTGGTCGCGC-3'). The *flgG* and *motAB* PCR fragments were digested with Ncol and Xbal and cloned into pTRC99a (Amersham Pharmacia Biotech) resulting in pBMM800 and pBMM802, respectively. The integrity and accuracy of all plasmid clones was verified by DNA sequencing.

**Biofilm formation**

The capacity of *P. luminescens* to form biofilms was assessed by measuring bacterial attachment to a plastic surface [2]. Strains were grown overnight in LB broth, diluted to OD₆₀₀= 0.05 in fresh LB and 200 μl of the cell suspension was aliquoted in triplicate, into the wells of a Costar® polypropylene (PP) 96-well microtitre plate. The plates were sealed with a gas permeable membrane and incubated, without shaking in a saturated environment to prevent evaporation, at 30°C. At the appropriate time the planktonic cells were removed by aspiration and the wells were washed with 1× phosphate buffered saline (PBS). To observe biofilm formation 250 μl of 0.1% (w/v) crystal violet (CV) was added to each well and the plates were incubated at room temperature for 20 min before rinsing 3 times with 1 × PBS. To quantify biofilm formation the CV was dissolved in 250 μl of 95% ethanol and the CV concentration was determined by measuring the OD₅₉₅ using a Genios (Tecan) plate reader.

**Pathogenicity assays**

The pathogenicity of *P. luminescens* was assessed using *Galleria mellonella* larvae (the Greater Wax Moth) as the

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**Table 1: Primers used for the construction of the Δ*flgG* and Δ*motAB* mutants**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ – 3’)</th>
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<tbody>
<tr>
<td>KO1*flgG</td>
<td>ATTATGCACTGCCGATAGAATACCTACCTCTCTGTCGCG</td>
</tr>
<tr>
<td>KO2*flgG</td>
<td>ATTATAGACTGCGCAGTAAACCGATCGACGTCTGCAG</td>
</tr>
<tr>
<td>KO3*flgG</td>
<td>GAGGTATAGGTCATCAGTCTGGTTTTATCCTCCTGTCTC</td>
</tr>
<tr>
<td>KO4*flgG</td>
<td>GGATAAAAACCGATGATACCTAATCTGCAATACTCAG</td>
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</tr>
<tr>
<td>KO6*flgG</td>
<td>GTGCCTGGGTGTAACAGCACGG</td>
</tr>
<tr>
<td>KO1*motAB</td>
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<tr>
<td>KO6*motAB</td>
<td>CGGCGCGTTGCAGCTGCGATCCCGC</td>
</tr>
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</table>

* restriction sites are underlined
In vitro symbiosis assays
An aliquot of 50 μl of an overnight culture diluted to an OD_{600} = 1.0 of the appropriate bacteria was spread, in a Z pattern, onto the surface of a lipid agar plate and incubated for 24 h at 30°C. The plates were incubated at 30°C for 3 days before adding 50 surface-stabilised IJ nematodes to the bacterial biomass. Nematodes were surface-stabilised by washing in a solution (0.4% (w/v)) of hyamine (Sigma). Nematode recovery was assessed 7 days after addition of IJs by counting the number of hermaphrodites on the lipid agar plate. The new generation of IJs migrated to the lid of the Petri dish and, after 21 days, these nematodes were collected, by washing the lid with PBS to a final volume of 50 ml, and the number of IJs present (i.e. the IJ yield) was determined. In competition assays the assays were the same except that the lipid agar plates were inoculated with equal numbers of the WT and mutant bacteria. At 3 and 21 days post-inoculation the bacterial biomass was scraped off the plate and the proportion of motile/non-motile bacteria was determined (as before). Colonisation levels in the IJ were determined by crushing single, surface-stabilised IJ nematodes in 100 μl 1 × PBS using a hand-held homogeniser and plating the homogenate onto LB (Rif) agar.

Authors’ contributions
CAE undertook all of the experiments described in this manuscript. DJC conceived of the study, designed the experiments and drafted the manuscript. All authors read and approved the final manuscript.

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