Biofilm Development on *Caenorhabditis elegans* by *Yersinia* Is Facilitated by Quorum Sensing-Dependent Repression of Type III Secretion

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Abstract

*Yersinia pseudotuberculosis* forms biofilms on *Caenorhabditis elegans* which block nematode feeding. This genetically amenable host-pathogen model has important implications for biofilm development on living, motile surfaces. Here we show that *Y. pseudotuberculosis* biofilm development on *C. elegans* is governed by *N*-acylhomoserine lactone (AHL)-mediated quorum sensing (QS) since (i) AHLs are produced in nematode associated biofilms and (ii) *Y. pseudotuberculosis* strains expressing an AHL-degrading enzyme or in which the AHL synthase (*ypsI* and *ytbI*) or response regulator (*ypsR* and *ytbR*) genes have been mutated, are attenuated. Although biofilm formation is also attenuated in *Y. pseudotuberculosis* strains carrying mutations in the QS-controlled motility regulator genes, *flhDC* and *fliA*, and the flagellin export gene, *flhA*, flagella are not required since *fliC* mutants form normal biofilms. However, in contrast to the parent and *fliC* mutant, *Yop* virulon proteins are up-regulated in *flhDC* and *fliA* and *flhA* mutants in a temperature and calcium independent manner. Similar observations were found for the *Y. pseudotuberculosis* QS mutants, indicating that the *Yop* virulon is repressed by QS via the master motility regulator, *flhDC*. By curing the *pYV* virulence plasmid from the *ypsI*/*ytbI* mutant, by growing *YpIII* under conditions permissive for type III needle formation but not *Yop* secretion and by mutating the type III secretion apparatus gene, *yscJ*, we show that biofilm formation can be restored in *flhDC* and *ypsI*/*ytbI* mutants. These data demonstrate that type III secretion blocks biofilm formation and is reciprocally regulated with motility via QS.

Introduction

The human pathogenic *Yersinia* (*Y. pseudotuberculosis, Y. enterocolitica* and *Y. pestis*) share a high degree of DNA identity, but cause distinct diseases ranging from enterocolitis (*Y. enterocolitica* and *Y. pseudotuberculosis*) to pneumonic, bubonic or septicemic plague (*Y. pestis*). Essential for the virulence of all pathogenic *Yersinia*, is the ~70-kb *pYV* virulence plasmid, which encodes the *Yop* virulon. This consists of a type III secretion system which enables *Yersinia* to inject multiple *Yop* effector proteins directly into the cytosol of eukaryotic cells and so subvert host cell signalling pathways (for reviews see [1–3]). *Yop* virulon genes are tightly regulated by environmental conditions and in particular, temperature (only expressing at 37°C) and Ca²⁺ concentration [reviewed in [4]].

*Y. pestis* and *Y. pseudotuberculosis* are capable of forming biofilms around the anterior and along the surface of the nematode *Caenorhabditis elegans* [5,6]. However, biofilm formation is strain-dependent and a study of over 40 different *Y. pseudotuberculosis* strains showed that some formed biofilms on *C. elegans* but not on abiotic polystyrene surfaces and vice versa [6]. No relationship was observed between strains forming biofilms on *C. elegans* and those that formed biofilms on polystyrene surfaces. These findings suggest that biofilm development on the living surface of *C. elegans* is different from that on an abiotic surface such as polystyrene.

*Y. pestis* is transferred between mammalian hosts by a flea borne vector that feeds on blood. The *hmsHFRS* operon is key to the colonisation and blockage of the flea proventriculus which results from the accumulation of biofilm [7–9] and *hmsHFRS* mutants of both *Y. pestis* and *Y. pseudotuberculosis* fail to form biofilms on *C. elegans*. Since *C. elegans* has been thoroughly studied at the genetic level and orthologous genes frequently studied in human health and disease, the *C. elegans/Yersinia* model can be used to identify genetic features of both the pathogen and the host that contribute to biofilm-mediated interactions between bacteria and invertebrates. These in turn have interesting implications for both the *Yersinia/Ileum* and human biofilm-centred infections. Although there are some limitations, the importance of *C. elegans* as a model organism for investigating prokaryotic/eukaryote interactions should not be overlooked given that nematodes are the most abundant animals on the Earth [10].
Author Summary

Many Gram-negative bacteria communicate by producing and sensing the presence of chemical signal molecules such as the N-acylhomoserine lactones (AHLs). Bacterial cells use AHLs to convey information about their environment, metabolism and population size. This type of chemical signalling is called ‘quorum sensing’ (QS) and is often used by pathogenic bacteria to promote acute or chronic infections through the control of motility, toxins, tissue degrading enzymes and surface-associated biofilms. *Yersinia pseudotuberculosis* is a human pathogen which forms biofilms on the surface of the nematode worm, *Caenorhabditis elegans*. This offers a simple means for investigating biofilm development on living tissues and can be used to identify genetic features of both the pathogen and the host that contribute to biofilm-associated infections. We have discovered that quorum sensing is required for *Y. pseudotuberculosis* biofilm formation on *C. elegans* through a regulatory pathway which involves the master motility regulator protein (FlhDC) reciprocally controlling bacterial swimming and the construction of a specialized secretion needle that delivers proteins into mammalian cells to disrupt their normal activities.

Although *Y. pseudotuberculosis* does not readily colonise fleas, biofilm formation may alternatively be involved in the prevention of predatory feeding as has been noted for other soil bacteria [11]. Whether the bacteria-invertebrate biofilm relationship is bacterially driven or is a two way interactive process between the bacteria and nematode is not fully understood. It has however been postulated that nematodes accumulate the bacterially derived extracellular matrix (ECM) passively by virtue of their movement through a lawn of bacteria [12] and there is evidence to show that biofilms do not accumulate on the surface of non-motile *C. elegans*. This implies that a prerequisite for biofilm formation is nematode translocation which provides the necessary contact between bacteria and nematode [12]. However, *Y. pseudotuberculosis* is unable to form biofilms on a number of motile *C. elegans* mutants such as *srf-3* and *bh-1* [6] and *sah-1*, *sah-2* and *sah-3*. Conversely many natural strains of *Y. pseudotuberculosis* fail to form biofilms on *C. elegans* as do a number of *Y. pseudotuberculosis* strains with mutations in lipopolysaccharide biosynthesis, signal transduction and *hms* genes [6]. Such findings imply the existence of an adaptive interaction between the nematode and the bacterium rather than simply the passive adherence of bacterially derived ECM [6].

Bacteria possess multiple integrated sensory systems that govern adaptation to environmental challenges including the local cell population density. Such population-dependent adaptive behaviour often takes the form of perception and processing of chemical information and is termed quorum sensing (QS). For many Gram negative bacteria this involves the use of self-generated diffusible signal molecules such as the N-acyl homoserine lactones (AHLs). These are usually synthesised and sensed *via* members of the LuxI AHL synthase and LuxR response regulator protein families respectively. QS enables bacteria to determine, by monitoring the concentration of a signal molecule, when the number of individuals in the population are sufficient (a quorum) to make a collective ‘decision’ to alter their behaviour in response to environmental challenges [13–16]. Such behavioural decisions impact on bacterial motility, secondary metabolism, virulence, and biofilm development [17].

*Y. pseudotuberculosis* produces four major AHLs via a QS system consisting of two genetic loci termed *ypsR/ypsI* and *ybrR/ybrI* which control cell aggregation/floculation and swimming motility [18,19]. This system is organized hierarchically with *YpsR* and its cognate AHLs regulating *ybrR* and *ybrI* as well as *ypsR* and *ypsI*. The *YpsR*/*YpsI* and *YbrR/YbrI* QS system in turn fine tunes swimming motility by governing the expression of two key regulators of the motility cascade, namely *flhDC* and *fliA* [19].

AHL-dependent QS also controls motility in *Y. enterocolitica* [20]. *Y. pestis* produces a similar range of AHLs to *Y. pseudotuberculosis* [21] and retains an analogous QS system [22,23]. However the relationship between QS and regulators of the motility cascade such as *flhDC* or *fliA* may be different in *Y. pestis* when compared with *Y. pseudotuberculosis* or *Y. enterocolitica* because *Y. pestis* is non-motile because of a frame-shift mutation in the motility master regulator *fliD* [24].

There is considerable evidence to show that AHL-dependent QS plays a significant role during the biofilm mode of growth on an abiotic surface since AHL production has been detected in glass and metal surface associated biofilms produced by bacteria such as *Pseudomonas aeruginosa* [25] and *Aeromonas hydrophila* [26]. Furthermore, in a variety of bacteria, QS controls the target genes required for different stages of biofilm development from adherence and aggregation to maturation and dispersal (for review see [27]). In addition QS determines the physiological response of biofilm communities to antimicrobial agents and host defences [28,29].

In the present paper we sought to determine whether biofilm formation by *Y. pseudotuberculosis* on a living motile surface i.e. on *C. elegans* is an interactive, QS-dependent process. The results obtained revealed that QS in *Y. pseudotuberculosis* reciprocally regulates the *C. elegans* biofilm phenotype with type III secretion via the major motility regulators *flhDC* and *fliA*. Consequently the induction of type III secretion attenuates biofilm formation on *C. elegans* which can be restored in a QS mutant either by curing the pYV virulence plasmid from the *ypsI/ybrI* mutant, by growing YpIII under conditions permissive for type III needle formation but not Yop secretion or by mutating the type III secretion apparatus gene, *yscJ*, a key component of the type III injectosome.

Results

*Y. pseudotuberculosis* produces AHLs when growing as a biofilm on the surface of *C. elegans*

When *C. elegans* is infected with *Y. pseudotuberculosis* YpIII harboring the gfp-plasmid pSB2020 and examined by confocal microscopy, the bacterial microcolonies fluoresce green and are embedded in an ECM which fluoresces red (yellow when both bacteria and matrix are combined) (Figure 1A) when labelled with WGA-R consistent with the presence of bacterially generated N-acetyl-D-glucosamine [12]. An orthogonal image of Figure 1A showing the depth of the biofilm in x and y planes can be seen in Figure S1A. After 48 h incubation the biofilms on *C. elegans* became highly resistant to WGA-R labelling and only stained red on the outer surface while the inner mass remained green (compare Figure 1A with Figure 1B). In common with bacterial biofilms formed on abiotic surfaces [30], the *Yersinia* biofilm on *C. elegans* also contains extracellular DNA as revealed by DAPI staining (Figure 2A).

To determine qualitatively whether AHLs are produced in the biofilms which accumulate on the surface of *C. elegans*, the biofilm matrix from heavily infected nematodes grown in the presence of *Y. pseudotuberculosis* for 24 h was extracted into dichloromethane and the extracts analysed using the AHL bioreporter *C. violaceum*.
CV026 in a well plate overlay assay [31]. As negative controls, AHL extractions were also carried out on nematodes which had been grown on E. coli OP50 and from the cell pellet of an overnight Y. pseudotuberculosis culture. Culture supernatant from the latter served as a positive control. Figure 3A (i) shows a purple halo of violacein around the agar well which contained the concentrated nematode extract taken from worms infected with parent Y. pseudotuberculosis. A similar result was obtained for the positive control (Figure 3A iv) while no violacein was observed around the negative control wells. Taken together these data indicate that AHLs are produced by Y. pseudotuberculosis growing as biofilms on the surface of C. elegans.

Figure 1. Y. pseudotuberculosis QS mutants are attenuated for biofilm formation on C. elegans. (A) Confocal image showing C. elegans heavily infected with Y. pseudotuberculosis YpIII embedded in a biofilm ECM which surrounds the anterior end of C. elegans and is spreading to other areas of the worm surface. Green, Gfp-labelled Y. pseudotuberculosis red, WGA-R binding to the ECM yellow, red and green overlay. (B) Confocal image of a Y. pseudotuberculosis YpIII biofilm on C. elegans after 48 h in which only the outer surface of the ECM stains with WGA-R which no longer penetrates deep into the biofilm. (C) C. elegans infected with the Y. pseudotuberculosisypsI/ytbl double mutant.

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Figure 2. Y. pseudotuberculosis biofilm ECM on C. elegans contains extracellular DNA. (A) The ECM fluoresces blue when stained with DAPI consistent with the presence of extracellular DNA. (B), (C) and (D) show the same image labelled with WGA-R (B; red), Gfp-labelled YpIII (C; green) and an overlay image (D) of the three fluorescent labels.

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Figure 3. AHLs are produced in Y. pseudotuberculosis YpIII biofilms on C. elegans. (A) C. violaceum AHL plate assay showing that AHLs are present in a Y. pseudotuberculosis biofilm growing on C. elegans. (i) Y. pseudotuberculosis YpIII biofilm extract harvested from C. elegans; (ii) extract from nematodes grown on E. coli OP50; (iii) cell pellet extract from an overnight liquid culture of Y. pseudotuberculosis and (iv) extract of an overnight liquid culture of Y. pseudotuberculosis YpIII. The AHL levels collected from the biofilm appear to be present at lower levels than in the culture supernatant. (B) Confocal image showing Y. pseudotuberculosis YpIII transformed with the AHL reporter, pJBA89 fluorescing green in response to AHLs in the biofilm. Red and yellow represent WGA-R stain of the ECM and the overlay of red and green respectively.

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To confirm that AHLs are synthesised in situ in the biofilms, *Y. pseudotuberculosis* was transformed with the *gfp*-biosensor, pJB89 which fluoresces green in the presence of AHLs [32]. When infected with *Y. pseudotuberculosis* pJB89 the characteristic biofilms which form on the surface of *C. elegans* after 24 h show green fluorescent *Y. pseudotuberculosis* pJB89 embedded in the red WGA-R labelled biofilm matrix (Figure 3B) which were indistinguishable from those presented Figure 1A.

Quorum sensing regulates biofilm development on the surface of *C. elegans*

Since AHLs were detected in the biofilms formed on *C. elegans*, we used two approaches to determine whether QS was required for biofilm development on the nematode surface. Firstly, we exploited the lactonase, AiiA which hydrolyses the ester bond within the AHL homoserine lactone moiety generating the corresponding, inactive, N-acylhomoserine compound [33]. When aiiA is introduced into *Y. pseudotuberculosis* on the pSU18 derivative pSA236, the AHLs produced are hydrolysed, so generating an AHL-negative phenotype [19]. By comparing the parent YpIII strain with either the pSU18 control vector or pSA236, we evaluated the contribution of AHL-dependent QS to biofilm development. For these experiments, a biofilm severity incidence was calculated for the infected *C. elegans* population after 24 h incubation. Each nematode was assigned a score between 0 and 3 related to the severity of biofilm accumulation (examples of scores 0 and 3 can be taken from the biofilms shown in Figure 1A and C; and scored of 1 and 2 from Figure S1 B and C respectively). These assays revealed that *Y. pseudotuberculosis* and *Y. pseudotuberculosis* pSU18 had biofilm severity indices of 77.3% and 62.0% respectively. When *C. elegans* infected *Y. pseudotuberculosis* pSU18 were compared to nematodes infected with *Y. pseudotuberculosis* pSA236 the biofilm severity incidence was reduced to 38.7% (p < 0.05 and n = 3 respectively) (Figure 4A).

Secondly we carried out *C. elegans* infection assays using *Y. pseudotuberculosis* YpIII QS mutants transformed with the constitutive *gfp*-plasmid, pSB2020. These included an AHL negative mutant in which both AHL synthase genes *ypsI* and *ypsR* have been disrupted and a second double mutant in which the two QS response regulators, *ypsR* and *ytbR* have been disrupted [18,19]. When compared with the parent *Y. pseudotuberculosis* YpIII strain (Figure 1A), biofilm development was severely delayed in the *ypsI*/*ytbR* double mutant formed little or no biofilm (compare Figure 1A and 1G). Similar results were obtained for the *ypsR*/*ytbR* double mutant (data not shown). In addition, nematodes grown on YpIII, in contrast to those grown on *E. coli* OP50, exhibit exaggerated body bends (Figure 5), are unable translocate within 1.5 h and by 5 h become moribund. In contrast, *C. elegans* infected with either the *ypsI*/*ytbR* mutant or the *ypsR*/*ytbR* mutant translocate normally and make tracks in the agar which are identical to those presented in Figure 5A and only began to show signs of aberrant movement 3–4 h post infection. After 96 h growth, both the *ypsI*/*ytbR* and *ypsR*/*ytbR* mutants formed severe biofilms on the nematodes. In addition, we calculated a biofilm severity incidence for each yersinia strain. Figure 4B shows that after 24 h there is an ~3 fold reduction in the amount of biofilm on nematodes infected by the *ypsI*/*ytbR* double mutant compared with the parent (32% compared with 89%; p = 0.011 n = 4). Similar results were obtained for the *ypsR*/*ytbR* double mutant (data not shown). Genetic complementation of the *ypsI*/*ytbR* mutant with pSA291 (Figure 4C) partially restored the biofilm severity incidence to that of the parent strain (Parent pHG327 (82%) compared with the *ypsI*/*ytbR* mutant pHG327 (35%) (p = 0.001 n = 3) and *ypsI*/*ytbR* mutant pSA291 (60%) compared with *ypsI*/*ytbR* mutant pHG327 (35%) (p < 0.05 n = 3).

These data demonstrate that the loss of AHL synthesis either via enzyme-mediated inactivation or by mutagenesis of the AHL synthases results in the attenuation of biofilm formation on *C. elegans*. Consequently QS is pivotal to the timing and severity of biofilm development on *C. elegans*.

Flagellar-mediated motility is not required for biofilm development on *C. elegans*

Since the *ypsR*/*ypsI* and *ytbR*/*ytbI* loci are both involved in the regulation of motility via *flhDC* and *flhA* which code for the motility master regulator and flagellar specific sigma factor respectively [19], we sought to determine whether these downstream regulators contribute to the *Yersinia/C. elegans* biofilm phenotype. Figure 6A shows that the *flhDC* mutant was impaired in its ability to form biofilms on the surface of *C. elegans* (biofilm severity incidence for the parent of 57.5% compared to 24.7% for the *flhDC* mutant (p < 0.05, n = 3) and genetic complementation of *flhDC* using pSA220 increased the biofilm severity to 61.6% when compared with the *flhDC* mutant (p < 0.02, n = 3). Figure 6B shows that the biofilm severity incidence for the *flhA* mutant was also reduced when compared with the parent (p < 0.05, n = 3). Since both regulators control swimming motility and as *flhDC* and *flhA* mutants are non-motile, these data suggested that biofilm formation may depend on flagellar-mediated motility. To explore this possibility, we first constructed a flagellin-negative strain by mutating the flagellin structural gene, *flhC*. This non-motile mutant formed biofilms on nematodes which were indistinguishable from the parent *Y. pseudotuberculosis* strain (Figure 6C and data not shown). Consequently, flagellar-mediated motility is not a necessary pre-requisite for biofilm formation on *C. elegans*. However, in *Y. enterocolitica*, the flagellar type III secretion apparatus may also secrete non-flagellar proteins termed ‘Fops’ (for Flagellar outer proteins) such as the phospholipase, YplA [34]. Since flagellar structural mutants still secrete Fops, we constructed a *flhA* mutant since this gene codes for a structural component of the flagellar protein export apparatus [35] and *flhA* mutants have been reported not to secrete Fops [34]. In common with the *flhDC* and *flhA* mutants and when compared to the parent, the *flhA* mutant exhibited attenuated biofilm formation (Figure 6B) (p < 0.05, n = 3), a finding which implies a possible role for a secreted protein(s).

To determine whether any secreted proteins could be involved in biofilm development on *C. elegans*, we first examined the extracellular protein profiles of the *Y. pseudotuberculosis* *flhDC*, *flhA*, *flhC* and *flhD* mutants grown overnight in LB$_{mnop}$ at 30°C. Figure 7 shows that compared with the parent strain and *flhC* mutant, numerous proteins are up-regulated in each of the other motility mutants. MALDI-TOF MS analysis identified three of the major protein bands a1-YopM, a1-YopH/1H (41/51 kDa), these two proteins often co-migrate and could not be distinguished by MALDI-TOF sequencing, LeV (37 kDa) and YopN (32 kDa) all of which are encoded in the pYV virulence plasmid and secreted by the Ysc-Yop type III secretion system. Two further up-regulated proteins were identified as KatY and GroEL which are not related to the Yop virulon (Figure 7).

Quorum sensing represses type III secretion in *Y. pseudotuberculosis*

In contrast to the YpIII parent strain which only secretes Yops at 37°C in the absence of Ca$^{2+}$ both *flhDC* and *flhA* mutants clearly secrete Yops at 30°C in the presence of Ca$^{2+}$. Since both of these
motility regulators are controlled by QS in *Y. pseudotuberculosis* [19], these data suggested that elements of the Yop virulon are also likely to be QS-controlled. Figure 8 shows that when grown in LBops at 30°C overnight, at least 4 extracellular proteins are up-regulated in the *ypsI/ytbI* and *ypsR/ytbR* double mutants compared with the parent strain. The same proteins are also up-regulated in the *ypsR*, *ytbR* and *ytbI* single mutants whereas the *ypsI* mutant exhibits the same profile as the parent strain. MALDI-TOF MS analysis identified the proteins as YopM/YopH, FliC, LcrV and YopN. These proteins were also present in supernatants from the same mutants after growth at 37°C in LBops but absent from the parent and *ypsI* mutant (Figure S2). In contrast, Yop proteins were absent from the supernatants of all of the strains grown at 22°C although two proteins, the flagellar capping protein (FliD; 48.6 KDa) and flagellin (FliC; 45 KDa) were up-regulated (data not shown).

The pYV plasmid inhibits biofilm formation by the *Y. pseudotuberculosis* *ypsI/ytbI* and *flhDC* mutants

The attenuation of biofilm formation on *C. elegans* observed for both the motility and QS mutants in conjunction with the elevated secretion of Yop virulon proteins at non-permissive temperatures raised the possibility that induction of type III secretion blocks...
biofilm development. Consequently, we predicted that biofilm formation would be restored in *Y. pseudotuberculosis* *ypsI/ytbI* and *flhDC* mutants cured of the pYV plasmid. To explore this hypothesis, we cured the pYV plasmid from the parent, *ypsI/ytbI* and *flhDC* mutants by repeated selection on CRMOX agar plates. The presence or absence of the pYV plasmid had no effect on the ability of the *Y. pseudotuberculosis* YpIII parent strain to form a biofilm on *C. elegans* (Figure 9A). However when similar experiments were performed using the *ypsI/ytbI* double mutant (Figure 9A and compare with Figure 4B) or *flhDC* (data not shown) cured of pYV, biofilm formation on *C. elegans* was restored to parental strain levels when compared with the biofilm levels observed on the *ypsI/ytbI* pYV+ double mutant (*p* = 0.01, *n* = 3).

These data suggest that under these conditions, AHL-mediated QS represses the expression of a pYV gene(s) which would otherwise prevent biofilm formation.

To gain further evidence in support of a biofilm inhibitory role for pYV Yop virulon component(s), *C. elegans* was infected with the parent *Y. pseudotuberculosis* grown in LB mops MOX, conditions which promote Yop secretion (i.e. 37°C in the absence of Ca²⁺) rather than in LB mops at 30°C in which Yops will not be secreted. These seed cultures were then transferred to NGM plates containing MgCl₂ and sodium oxalate to chelate Ca²⁺. Under such pre-conditions, the type III system is induced and no biofilms were formed on *C. elegans* (data not shown) providing additional support that induction of the Yop virulon prevents biofilm formation on *C. elegans*.

Biofilm formation on *C. elegans* is inhibited by induction of the type III injectisome

To demonstrate unequivocally that the inhibition of biofilm formation on *C. elegans* observed for the *Y. pseudotuberculosis* *ypsI/ytbI* mutant depends on the induction of functional type III secretion system rather than other genes present on the pYV plasmid, we modified the *ypsI/ytbI* mutant by mutating *yscJ*. This gene codes for a key component of the Ysc injectisome required for the assembly of a functional type III secretion apparatus [36]. Cell free culture supernatants taken from the *ypsI/ytbI*/*yscJ* triple mutant grown in LB MOX at 30°C were examined by SDS-PAGE. This confirmed that, in contrast to the *ypsI/ytbI* mutant, Yop proteins were no longer secreted (data not shown). Yop secretion in the triple mutant grown under these conditions could however be restored by complementation with a plasmid-borne copy of *yscJ*.

Figure 6. *Y. pseudotuberculosis* strains with mutations in *flhDC*, *fliA* or *flhA* but not *fliC* are attenuated for biofilm formation. Biofilm severity indices are shown for *flhDC* and the complemented *flhDC* mutant (A), *fliA* and *fliA* (B) and *fliC* (C).

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Figure 7. SDS-PAGE protein profiles of cell free supernatants prepared from *Y. pseudotuberculosis* YpIII parent, *flhDC*, *fliA*, *flhA* and *fliC* mutants grown at 30°C. The up-regulated proteins YopN, YopM/H, LcrV, KatY and GroEL were identified by MALDI-TOF MS. Molecular masses of the marker proteins are in kDa.

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In the *C. elegans* biofilm assays, the biofilm severity index of the *ypsI/ytbI/yscJ* triple mutant was ~4-fold higher than that of the *ypsI/ytb* double mutant (*p* = 0.05, *n* = 3) and comparable with that of the parent strain (Figure 9B). When the triple mutant was compared to its complemented counterpart containing a functional copy of *yscJ* (on plasmid pHG::*yscJ*), biofilm severity was reduced ~two-fold (*p* < 0.01, *n* = 3) back to levels comparable with the *ypsI/ytb* double mutant (Figure 9B). These results are consistent with a role for the type III injectisome in preventing biofilm development on *C. elegans* and demonstrate that either the type III needle or the secreted Yop proteins or both prevent biofilm development on *C. elegans*. To attempt to differentiate between these three possibilities, we grew the *Y. pseudotuberculosis* *YpIII* parent strain at 37°C in the presence of calcium which results in type III needle assembly but not Yop secretion [37]. This is because Ca²⁺ prevents Yop effector secretion even in the presence of a fully formed injectisome. *YpIII* was then subcultured onto NGM medium supplemented with calcium. When pre-cultured under these conditions and used to infect *C. elegans* at 22°C, *Y. pseudotuberculosis* *YpIII* failed to form a biofilm on *C. elegans*. The infected worms were indistinguishable from that shown in Figure 1C suggesting that the type III needle rather than the Yop effectors was responsible for preventing biofilm development.

**Discussion**

On abiotic surfaces, bacterial biofilm formation is generally considered as a step-wise process initiating from individual cells adhering to a substratum leading to microcolony formation, biofilm maturation and finally dispersal to new sites [38–42]. Although the nature and development of biofilms formed on biotic surfaces have not been as thoroughly investigated, biofilm development by *Y. pseudotuberculosis* on *C. elegans* involves attachment and maturation stages and the ECM contains both carbohydrate and extracellular DNA. Whether the DNA present in the biofilm is bacterial or nematode-derived has yet to be established. However, the WGA-stained carbohydrate present in the biofilm appears to be bacterially-derived since it is present in the lawns of *Y. pseudotuberculosis* prior to the addition of nematodes which are not labelled by WGA [43]. The WGA-stained ECM carbohydrate could be either peptidoglycan which contains N-acetyl glucosamine in the sugar backbone [44] or polymeric N-acetyl-D-glucosamine or both. *Y. pestis* strains with mutations in the *hmsHFRS* locus, which is responsible for the biosynthesis of a poly β-1,6-N-acetyl-D-glucosamine-like polysaccharide [45], are defective for biofilm accumulation on *C. elegans* implying that this exopolysaccharide plays an essential role. An intact *hmsHFRS* is also required for biofilm formation on *C. elegans* by both *Y. pseudotuberculosis* and *Xenorhabdus nematophila* [46].

**Figure 8.** SDS-PAGE protein profiles of the *Y. pseudotuberculosis* parent and the QS mutants prepared from cell-free supernatants grown at 30°C. YopN, YopH/M, LcrV, and FliC were identified by MALDI-TOF MS. Molecular masses of the marker proteins are in kDa. doi:10.1371/journal.ppat.1001250.g008

**Figure 9.** Impact of pYV and type III secretion on biofilm formation by *Y. pseudotuberculosis* *YpIII* on *C. elegans*. Biofilm severity indices are shown for (A) *YpIII* and the *ypsI/ytb* mutant with or without pYV and (B) *YpIII* and the *ypsI/ytb* mutant compared with the *ypsI/ytb*/*yscJ* triple mutant and the triple mutant complemented with a plasmid borne copy of *yscJ*. doi:10.1371/journal.ppat.1001250.g009
Apart from the hmsHFRS genes, other yersinia genes currently known to be required for biofilm formation on C. elegans include two genes involved in LPS biosynthesis, two genes of unknown function and a potential hybrid two component regulatory protein [6]. Both RecA (a phosphorelay accessory protein which functions in concert with the response regulator, RecB) and PhoP negatively regulate the formation of Y. pestis biofilms on nematodes [47] while the action of PhoP appears to be mediated at least in part by the down-regulation of HmsT [48]. This is interesting since HmsT is a cyclic di-γ-cysteine (c-di-GMP) synthetase and c-di-GMP metabolism plays an important role in biofilm formation in many different bacteria including V. cholerae [49,50].

Depending on the organism, QS may be involved in the early attachment or later maturation stages of biofilm development on abiotic surfaces [27]. In pathogens such as V. cholerae, QS is responsible for controlling the expression of key components of the biofilm extracellular matrix including exopolysaccharides and extracellular DNA release as well as the refractory nature of biofilms to host defences and antimicrobials [27]. The contribution of QS to yersinia biofilm development on C. elegans has not previously been investigated although for Y. pseudotuberculosis, QS controls cell aggregation [a type of suspended biofilm] in liquid culture [18]. A V. cholerae strain with combined mutations in ypsR/ypsI and ytbR/ytbI and luxS formed a similar biofilm on glass cover slips to the parental strain which could not be distinguished by crystal violet or Congo red staining although a very mild defect was observed using confocal microscopy [51]. Here, for Y. pseudotuberculosis YpIII, we have shown that AHL-dependent QS is functional in biofilms formed on C. elegans by demonstrating (i) the presence of AHL signal molecules within the nematode-associated biofilm matrix and (ii) that YpIII strains in which AHL biosynthesis is abrogated either by expressing an AHL-inactivating enzyme in situ or by mutating the AHL synthetases (YpsI and YtbI) are attenuated for biofilm formation. Because Y. pseudotuberculosis YpIII does not form biofilms on polystyrene surfaces [6], these data indicate that the QS-dependent pathway for biofilm formation on C. elegans is different from that on abiotic surfaces. While QS signals have previously been identified in pseudomonas and aeromonas biofilms on abiotic surfaces [25,26] to our knowledge they have not previously been detected directly in biofilms growing on a living, biotic surface. AHLs have however been shown to be produced in the tissues of mice infected with Y. enterocolitica [52] although no evidence was presented for biofilm formation in this acute experimental infection model.

In Y. pseudotuberculosis, YpsRI and YtbRI form a QS hierarchy in which ypsR is auto-regulated and also controls the expression of ypsI, ytbI and ytbR; YtbR also regulates ytbI expression [19]. In common with the ypsI/ytbI double synthase mutant, the ypsR/ytbR double response regulator mutant was also attenuated for biofilm development on C. elegans. The ypsR/ytbR mutant however produces a similar AHL profile to that of the parent strain [19] and therefore AHL production per se is not required for biofilm formation. The intermediate level biofilms formed by the single ypsR, ytbR and ytbI mutants (data not shown) reflect the interdependent nature of the Y. pseudotuberculosis QS system while the lack of biofilm attenuation observed for the ypsI mutant suggested that the ACHs synthesized via YtbI are primarily responsible for the biofilm phenotype observed.

A number of Gram-negative bacterial species rely on flagellar-mediated motility for specific stages of biofilm formation [38]. For example, in E. coli, mutations which lead to either the loss of flagella or flagella function (which include flaC or flaD) are unable to form mature biofilms indicating that the presence of functional flagella is a pre-requisite for biofilm development in a PVC attachment model [33]. Similarly, non-motile yet flagellate P. aeruginosa PA01 flaK mutants are V. cholerae flaC or motA mutants cannot form biofilms on PVC surfaces [54,55]. Furthermore, in V. enterocolitica, mutations that abolish the structure or rotation of the flagellar greatly reduced biofilm formation in PVC microplate assays [56]. Thus, given the links between biofilm formation, flagella-mediated motility and the regulation of the two key motility regulators, flhDC and fliA by QS in Y. pseudotuberculosis [19], we investigated the contribution of motility to biofilm formation on C. elegans. Surprisingly, a Y. pseudotuberculosis flaC mutant formed similar biofilms to the parent strain indicating that on the nematode, the presence of flagellar is not a pre-requisite for biofilm formation. This provides further evidence to suggest that the biofilm developmental pathway on the living nematode surface is distinct from that occurring on an abiotic surface. Since flagellins are potent inducers of the innate immune response and are often considered as flags revealing the presence of bacteria [57], it may therefore be advantageous for Yersinia to repress their expression during growth on living surfaces.

Despite the lack of biofilm attenuation for the flaC mutant, non-motile strains with mutations in fliA, a structural component of the flagellar export apparatus as well as the motility cascade regulators, flhDC and fliA were significantly attenuated. Since QS governs the expression of key motility regulators [19] these data suggested that biofilm formation on C. elegans by Y. pseudotuberculosis was linked to QS via the motility cascade. As V. cholerae has a frameshift mutation in flhD, biofilm formation on C. elegans in V. cholerae may well be governed differently to Y. pseudotuberculosis. Y. enterocolitica secretes FOP proteins such as YplA via the flagellar type III secretion apparatus [34]. Consequently, we considered it possible that the loss of Y. pseudotuberculosis FOP proteins by mutation of the motility genes may have been responsible for biofilm attenuation. However, SDS-PAGE analysis of the extracellular protein profile of these strains did not reveal any novel FOP proteins but rather the presence of several proteins associated with the Yop virulon and type III secretion. In particular, LcrV which is associated with the tip of the injectisome and with pore formation across the host cell membrane, YopN, a plug considered to limit Yop effector translocation through the needle and YopH, a phosphotyrosine phosphatase effector protein which inhibits phagocytosis (reviewed by [2]). Our findings are consistent with observations made by [58] that deletion of flhDC resulted in the up-regulation of the yop regulon in Y. enterocolitica as a consequence of FlhDC-mediated repression of the Yop virulon regulator gene, virF.

Since QS in Y. pseudotuberculosis regulates flhDC and fliA [19] we also examined cell free supernatants of strains with mutations in the ypsR/ytbR loci for the up-regulation of Yop virulon proteins. Apart from the single ypsI mutant, which exhibited the parental phenotype, each of the QS mutants exhibited the same protein profile on SDS-PAGE as the flhDC and fliA mutants when grown at 30°C in the presence of Ca²⁺. Since both injectisome and Yop effector proteins were up-regulated, these data suggest that QS represses the Yop virulon via the actions of FlhDC on virF. In addition, it is clear that mutation of QS results in the loss of both the temperature and Ca²⁺ dependence characteristic of type III secretion in Yersinia. Thus in Y. pseudotuberculosis, QS positively regulates motility but negatively controls type III secretion indicating that both phenotypes are population dependent. This would suggest that in the planktonic phase at high population densities in the presence of eukaryotic target cells, Yop secretion would be shut down in favour of bacterial migration to new sites where a fall in QS signal concentrations would stimulate the resumption of Yop secretion.
With respect to the biofilm phenotype of the QS and motility mutants, the de-repression of type III secretion at temperatures below 37°C suggested that type III secretion blocked biofilm formation on C. elegans. Since the Yop virulen genes are located entirely on the pYV plasmid, we examined the biofilm phenotype of the plasmid-cured parent, ypoD/ytbI and fbdC mutants respectively. The loss of pYV from the parent Y. pseudotuberculosis strain had no impact on biofilm formation an observation which is fully in agreement with Joshua et al. (2003) [6] who examined both YpIII and a range of Y. pseudotuberculosis strains with or without the virulence plasmid. However the attenuation of biofilm formation observed for both the ypoD/ytbI and fbdC mutants could be overcome by curing pYV, a finding which implied that QS repression the expression of pYV encoded gene(s) which block biofilm formation in the presence of Ca\(^{2+}\) and at 22°C, the temperature at which the C. elegans assays are carried out. Additional support for these observations was obtained when seed cultures of the Y. pseudotuberculosis parent strain were grown under conditions permissive for Yop release (37°C in the absence of Ca\(^{2+}\)) and then transferred onto Ca\(^{2+}\)-free modified NGM plates at 22°C whereupon biofilms did not form on C. elegans.

To rule out the possibility that other genes located on the pYV plasmid were responsible for the biofilm phenotype rather than the presence of a functional type III secretion system, we introduced a ypoD/ytbI mutation into the ypoD/ytbI double mutant. The newly generated triple mutant resulted in the loss of type III secretion at 30°C in the presence of Ca\(^{2+}\) and the restoration of biofilm formation on C. elegans. This strongly implies that the presence of an intact injectosome blocks biofilm formation on C. elegans. However, these data alone could not determine whether the reduction in biofilm was due to the presence of an intact injectosome, extracellular Yops or both. Evidence to suggest that the type III injectosome rather than the Yop effectors were responsible for attenuating biofilm formation on C. elegans was obtained by first conditioning seed cultures of Y. pseudotuberculosis at 37°C in Ca\(^{2+}\) containing media prior to carrying out biofilm assays. We reasoned that the conditioned Y. pseudotuberculosis cells would possess intact injectosomes but would not release Yops [59–63]. Furthermore, the presence of Ca\(^{2+}\) in the NGM agar would continue to suppress Yop secretion during the biofilm assays. When biofilm assays were performed using pre-conditioned Y. pseudotuberculosis cells biofilm formation was suppressed. These data appear to preclude a requirement for extracellular Yops in order for biofilm formation to take place. The simplest explanation is that the presence of the fully formed needle acts as a physical barrier which blocks the interaction between a key, chromosomally encoded bacterial surface component and the nematode surface. This would also be consistent with the loss of biofilm formation which results from the mutation of a number of C. elegans surface-determining genes [64,65]. However, at this stage we cannot rule out the possibility that contact between the injectosome and C. elegans results in the repression of as yet unidentified genes required for biofilm formation.

**Materials and Methods**

**Strains and growth conditions**

The Y. pseudotuberculosis, Escherichia coli and C. elegans strains and the plasmids used in this study are listed in Table S1 and Table S2 respectively. To aid visualisation of Y. pseudotuberculosis in biofilm assays, the bacterial cells were transformed with pSB2020 [66] which constitutively expresses gfp3. To determine whether biofilm formation on C. elegans could be attenuated by AHL hydrolysis, Y. pseudotuberculosis YpIII was also transformed with the lactonase gene, aiiA on pSA236 as described before [19]. Except where stated, bacterial cultures were routinely grown with shaking at 200 rpm in L broth Lennox [67] or on agar plates containing the appropriate antibiotics buffered to pH 6.8 with Mops (3-N morpholino) propanesulfonic acid (YLBmops) to reduce alkaline hydrolysis of AHLs during bacterial growth [68]. To promote yop expression at 37°C some experiments were performed in YLBmops supplemented with MgCl\(_2\) (20 mM) and sodium oxalate (20 mM) as previously described [58]. Where required, pYV was cured from Y. pseudotuberculosis by the repeated sub-culture of white colonies onto Congo red-magnesium oxide (CRMOX) plates [69].

The C. elegans wild-type (N2 Bristol) strain was obtained from the Caenorhabditis Genetics Centre (University of Minnesota, St. Paul, MN) and maintained on modified NGM plates [70] lacking MgCl\(_2\), seeded with E. coli OP50 unless otherwise stated. For Yop induction assays NGM was supplemented with MgCl\(_2\) (20 mM) and sodium oxalate (20 mM) but CaCl\(_2\) was omitted.

**Y. pseudotuberculosis/C. elegans biofilm assay**

NGM plates were seeded with 1 ml of the appropriate Y. pseudotuberculosis strain grown overnight at 30°C unless otherwise stated. For some C. elegans biofilm experiments, NGM agar plates were modified by the addition of sodium oxalate (20 mM) and MgCl\(_2\) (20 mM) to promote Yop secretion. For the assays in which biofilm severity incidence was calculated, Y. pseudotuberculosis were spread evenly over the agar surface, dried to remove excess liquid and 20–30 young adult C. elegans were aseptically transferred to the seeded plates. After incubation for 22°C for 24 h (unless otherwise stated), the worms were examined under low magnification using a Nikon SMZ1000 microscope and biofilm accumulation was classed as level 0 if no biofilm formed (e.g. Figure 1C); level 1 indicating a small accumulation of biofilm around the anterior end of the worm (e.g. Figure S1B); level 2 denoted larger accumulations of biofilm around the anterior end of the worm with some pockets of biofilm spreading back from the head (e.g. Figure S1C); level 3 by large accumulations of biofilm around the anterior end of the worm which extended to other parts of the nematode body surface (e.g. Figures 1A and 2B). Confocal images of C. elegans were taken using a Zeiss LSM7100 inverted microscope. Repeat Z-stacks were taken at 5 μm intervals. The Zeiss Zen software package was used for image analysis. The level of biofilm accumulation on C. elegans was denoted as the biofilm severity incidence and was calculated according to the method of Tarr [71]: Biofilm severity incidence = \{\sum \text{level X number of samples in this level} \}/\text{highest level X total sample numbers} X 100%. All assays in which the level of biofilm severity was assessed were carried out double blind, with at least three or four replicates and each experiment was performed more than once. The error bars shown on figures 4, 6 and 9 represent the standard deviation from the mean and when necessary independent two-sample t-tests were performed with values for p and n given in the text and on histograms where appropriate. For some experiments the presence of the N-acetyl-D-glucosamine in the ECM of Y. pseudotuberculosis biofilms was demonstrated using a wheat germ agglutinin (WGA)-rhodamine (WGA-R) conjugate as described by [12]. Extracellular DNA present in the biofilms was stained with DAPI following the method of Vilain et al., [72] in which low concentrations of DAPI are demonstrated to label the extracellular biofilm matrix without penetrating the bacterial cell and staining the intracellular DNA.

To determine whether biofilm formation was attenuated when worms were infected with Y. pseudotuberculosis containing the AHL lactonase AiiA, aiiA was excised from pSA302 [19] as an EcoRI fragment and ligated into the E. coli plasmid pSA236 to give pSA302a. This plasmid 3day
fragment and then sub-cloned into the chloramphenicol resistant vector pSU18 [73] to give pSA236 which was transformed into Y. pseudotuberculosis. pSU18 was transformed into Y. pseudotuberculosis to act as a vector control.

DNA manipulations

Plasmids were isolated using the Promega Wizard system, agarose gel electrophoresis and standard methods for the preparation of competent cells, DNA ligation and electroporation were performed as previously described [20]. For the purification of DNA fragments from agarose gels, Qiagen DNA purification columns were used (Qiagen Ltd). Restriction endonucleases, DNA ligase and other DNA modification enzymes were used according to the manufacturers’ instructions (Promega).

AHL detection

C. elegans infected with Y. pseudotuberculosis were removed from 40 NGM plates in M9 wash solution [74]. The worms were washed, the pellet extracted into dichloromethane, reconstituted to 20 μl of acetonitrile and analysed using a well plate overlay assay using the C. violaceum CV026 biosensor which reports the presence of AHLs by producing the purple pigment violacein [31]. To detect AHLs produced in situ in biofilms on the surface of C. elegans, Y. pseudotuberculosis and the isogenic ypsI/ytbI double mutant were each transformed with the AHL biosensor, pJBA89 [32] which expresses gfp in the presence of AHLs. Infected worms were examined using fluorescent microscopy for the presence of green fluorescent bacteria within the biofilm matrix.

Construction of Y. pseudotuberculosis mutants

Y. pseudotuberculosis YpIII strains with deletions in fliA, flhA, flhC and yscJ were constructed as follows. The fliA mutant was constructed using a modified method of [75]. The primers used for mutant construction are listed in Table S3. Briefly, primer pairs flhA1up-F/flhA1up-R and flhA1down-F/flhA1down-R were used to amplify 510 and 511 bp fragments of the up- and downstream regions of flhA (positions 2069236 to 2069746 and 2070363 to 2070874 in the published Y. pseudotuberculosis IP 32953 genome sequence [76]). Primer flhA1up-R and flhA1down-F also contained 25 and 22 bp respectively of sequence homologous to the first 25 bp and last 22 bp of kanamycin from pUC4K [77]. The kanamycin cassette was amplified from pUC4K (Pharmacia) using primer km-F and km-R under the following PCR conditions: 95°C for 5 min followed by 30 cycles of 95°C for 30 s, 56°C for 30 s and 74°C for 1 min and ending with 74°C for 5 min. The second and third step PCR conditions were as follows: 95°C for 5 min followed by 30 cycles of 95°C for 30 s, 60°C for 30 s and 74°C for 2 min and ending with 74°C for 5 min. The strategy for constructing the flhA and yscJ mutants was similar to that of fliA. For flhA, primer pairs flhA1up-F/flhA1up-R and flhA1down-F/flhA1down-R were used to amplify the up- and downstream regions of flhA (positions 2017164 to 2017699 and 2019587 to 2020179 on the published IP 32953 Y. pseudotuberculosis genome sequence) whereas for yscJ, primer pairs YscJF-XbaI and YscJR-SalI were used to amplify the up- and downstream regions of yscJ (positions 59172 to 59743 and 60344 to 61135) on the published Y. pseudotuberculosis IP 32953 pYY virulence plasmid sequence. For flhA, primer pairs flhA1up-R and flhA1down-F each contained 19 bp of sequence homologous to the first 19 bp or last 19 bp of the kanamycin cassette from pUC4K whereas for yscJ, YscJP-R-Tet and YscJdown-F-tet contained 21 bp or 22 bp of sequence homologous to a tetracycline cassette which was amplified as a 1191 bp product from pBlue-tet (a source of the tetracycline cassette initially amplified from pBR322 using primers Tet1 and Tet2 [19] and cloned into pBlueScript as an Xhol fragment). All PCR conditions were the same as those for the construction of the flhC fragment.

To complement yscJ, primers YscJF-Xhol and YscJR-Sall were used to amplify an 842 bp product from Y. pseudotuberculosis (positions 59686 to 59703 on the IP32953 published sequence) which, after cloning into pBlueScript and sequencing was excised as a KpnI and PstI fragment and sub-cloned into the low copy number vector pHG327 [70]. The resulting plasmid, pHG::ycsJ was transformed into the Y. pseudotuberculosis ypsI/ytbI double mutant.

Colonies PCR was used to amplify a βC homologue from Y. pseudotuberculosis using the primers DC1 and DC2 and cloned into pGEMT/easy (Promega) to give pβC. Sequencing revealed the 1,515 bp fragment to have an open reading frame of 1,110 bp and predicted protein product of 396 amino acids that shared significant amino acid similarity to several Flh homologues and was subsequently termed flhCy (Genbank accession number AF244535). To construct a flhC mutant 616 bp was removed from pβIC using Csp45I and replaced with a kanamycin cassette from pUC4K (Pharmacia) as a blunt end fragment. The resulting construct was cloned into pDM4 as a SpeI-XbaI fragment (pDM βC-Km) and stably integrated into the chromosome of Y. pseudotuberculosis as previously described [18,19].

To complement the Y. pseudotuberculosis YpIII flhDC mutant [19] flhDC was amplified by PCR (primers FlhDF and FlhCR), cloned into pGEMT/easy (Promega) and the resulting pGEM::flhDC construct, pSA2290 was transformed into the Y. pseudotuberculosis flhDC mutant. The flhDC, flhA, fliC and flhC mutants were examined for motility using swim agar plate assays and microscopy and the presence of flagella proteins was determined by SDS-PAGE once isolated from 24 h overnight liquid cultures grown at 22°C as previously described [20,79].

SDS-PAGE and protein sequencing

Proteins present in 10 ml of cell-free supernatant taken from Y. pseudotuberculosis QS and motility mutants grown to the same OD600 (overnight in YLB at 22°C, 26°C and 37°C) were concentrated by trichloroacetic acid precipitation, subjected to SDS-PAGE and the relevant bands excised. After in-gel tryptic digestion, the resulting peptides were identified by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF)-MS sequencing as previously described [20].

Supporting Information

Figure S1 Orthogonal images of Figure 1A showing the Y. pseudotuberculosis YpIII biofilm depth in cross section through the x and Y planes (A). Examples of severity level 1 and 2 biofilms on the surface of C. elegans are shown in (B and C).

Figure S2 Protein profiles of supernatants taken from Y. pseudotuberculosis YpIII and the QS mutants grown at 37°C. Four up-regulated proteins were identified as YopM/H, LcrV, YopN and FlhC.

Table S1 Strains used in this study.

Table S2 Plasmids used in this study.
Acknowledgments

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References


The complete list of references is available in the PDF version of the article.


