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ARTICLE

Biofilm matrix disrupts nematode motility and predatory behavior

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Abstract

In nature, bacteria form biofilms by producing exopolymeric matrix that encases its entire community. While it is widely known that biofilm matrix can prevent bacterivore predation and contain virulence factors for killing predators, it is unclear if they can alter predator motility. Here, we report a novel "quagmire" phenotype, where *Pseudomonas aeruginosa* biofilms could retard the motility of bacterivorous nematode *Caenorhabditis elegans* via the production of a specific exopolysaccharide, Psl. Psl could reduce the roaming ability of *C. elegans* by impeding the slithering velocity of *C. elegans*. Furthermore, the presence of Psl in biofilms could entrap *C. elegans* within the matrix, with dire consequences to the nematode. After being trapped in biofilms, *C. elegans* could neither escape effectively from aversive stimuli (noxious blue light), nor leave easily to graze on susceptible biofilm areas. Hence, this reduced the ability of *C. elegans* to roam and predate on biofilms. Taken together, our work reveals a new function of motility interference by specific biofilm matrix components, and emphasizes its importance in predator–prey interactions.

Introduction

Bacteria colonize most natural surfaces and their hosts as biofilms. The encased community of bacterial cells produces its extracellular matrix that serves as a barrier from physicochemical factors [1], and allows bacterial differentiation and specialization [2, 3]. Living in biofilms can offer strong competitive advantages in the presence of various environmental stresses, such as predation, immune attack, and antimicrobials. Depending on the stimuli and gene regulatory networks, the biofilm matrix is highly complex and dynamic. For instance, the opportunistic pathogen *Pseudomonas aeruginosa* secretes varying compositions and levels of exopolysaccharides (Pel, Psl, and alginate), adhesion proteins (CdrA), extracellular DNA (eDNA), allowing it colonize both abiotic and biotic surfaces [4].

The production of biofilm matrix is mainly mediated by the c-di-GMP secondary messenger system, which is common in most gram-negative bacterial species [5]. Synthesis of c-di-GMP by diguanylate cyclases (DGCs) will lead to biofilm formation via loss of bacterial motility and induced production of exopolymeric matrix, whereas phosphodiesterase (PDE)-mediated degradation of c-di-GMP causes biofilm dispersal and production of specific virulence factors, such as exotoxin A from type III secretion system (T3SS) and rhamnolipids [6–9]. The redundancy of DGCs and PDEs in *P. aeruginosa* leads to fine-tuning the expression of various biofilm matrix components. For instance, production of the Pel and Psl exopolysaccharides is controlled by the *wsp* operon, with WspR as the DGC [10], whereas MucR controls alginate production [11]. PDEs, such as DipA, are involved in biofilm dispersal [12], while RocR mediates swarming motility [13].

In the natural environment, *Caenorhabditis elegans* are bacterivores that roam and feed on microbial biofilms growing on rotten fruit or plant biomass. As a model organism applicable to a multitude of research fields encompassing developmental biology, behavioral studies to infections, *C. elegans* are experimentally grown on bacterial lawns in media plates. While *Escherichia coli* OP50 are

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common food choices for *C. elegans*, *Yersinia* biofilms were previously shown to block the mouth and prevent bacterial uptake by *C. elegans*, resulting in the nematodes' death by starvation [14]. Furthermore, biofilms formed by *P. aeruginosa* [15] and *Salmonella* [16] could produce specific virulence factors, such as pyoverdine [17], that killed *C. elegans* after being internalized into the intestine. While these effects mainly revolved around *C. elegans* feeding and intestinal infection, it is unclear if biofilms can alter motility which is a key feature of *C. elegans*.

Here, we report for the first time that the biofilm matrix can alter *C. elegans* locomotion and its resulting behavior, herein termed as the "quagmire" phenotype. Using the *P. aeruginosa* mutant library of known components and regulators for biofilm matrix, we showed that Psl, a key exopolysaccharide present in the *P. aeruginosa* biofilm matrix, could impede nematode locomotion, which was adequately reflected in reduced velocity and roaming of *C. elegans*. Trapping of nematodes in the *P. aeruginosa* biofilm matrix significantly reduced the ability of *C. elegans* to either escape from a noxious blue-light repellent, or move toward the susceptible OP50 biofilms.

Taken altogether, our study suggests a novel mechanism by which biofilms employ to impede *C. elegans* movement, possibly to delay predation and boost survival. In the context of bacterial infections, our findings also suggest a plausible similarity in specific biofilm matrix components impeding immune cell migration.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Supplementary Table S1. *E. coli* DH5a strain was used for standard DNA manipulations. Luria–Bertani (LB) medium was used to cultivate *E. coli* and *P. aeruginosa* strains. For plasmid maintenance in *E. coli*, the medium was supplemented with 100 μg ml^{-1} ampicillin and 15 μg ml^{-1} gentamycin. For marker selection in *P. aeruginosa*, 30 μg ml^{-1} gentamycin, 30 μg ml^{-1} tetracycline, or 100 μg ml^{-1} carbenicillin was used, as appropriate.

Growth and maintenance of *C. elegans*

The bacterial lawn, such as *E. coli* OP50 and *P. aeruginosa* strains, was cultivated on nematode growth media (NGM) agar plates at 37 °C for 16 h. The laboratory *C. elegans* N2 nematode was transferred to the bacterial lawn on the NGM agar plates and cultivated at room temperature for 72 h to allow the population expansion.

C. elegans motility assay

All *P. aeruginosa* strains were first inoculated and grew in 2 ml of LB medium at 37 °C with shaking at 200 rpm for 16 h. After washing the overnight culture with 0.9% NaCl, the 30 μl of bacterial culture was transferred to NGM petri dish (3 cm diameter) for spreading with a bacteriological spreader. The culture was incubated overnight to allow biofilm growth. At least 50 individuals of *C. elegans* from 3 independent trials were transferred from the feeding plate to the center of the biofilm with titanium wire picker. Unless stated otherwise (such as L1 larvae and adults), L3 *C. elegans* were used for all experiments. The nematodes were given 5 mins for them to adapt to the fresh environment. Before recording the video, the plate was tapped for three to four times to stimulate the *C. elegans* roaming in the bacterial biofilm lawn.

Track, displacement, and velocity analysis

The 30-sec video clip of the locomotion of each single individual was recorded by a stereomicroscope (Nikon SMZ1270i, Japan) at ×10 magnification. The video clips were processed and analyzed with image processing software "ImageJ Fiji". The locomotion of *C. elegans* each frame was tracked using the manual tracking plugin. The track images were captured and the frame velocity was calculated by the software in μm per second. The frame displacement was calculated in pixels by the following formula:

$$\sqrt{\frac{(x_2 - x_1)^2}{(y_2 - y_1)^2}},$$

x_1 = the initial x-coordinate, x_1 = the x-coordinate in the next frame of x_1, y_1 = the initial y-coordinate, y_2 = the y-coordinate in the next frame of y_1.

Nematode escape assay

The overnight cultures of *P. aeruginosa* strains were inoculated and grown in 2 ml LB medium at 37 °C overnight with shaking at 200 rpm. The bacterial culture was centrifuged to collect the cell pellet. The cell pellet was resuspended with 50 μl 0.9% NaCl. The entire resuspended culture was then transferred to the center of the NGM plate to make a concentrated spot (diameter ~3 mm) for the growth of biofilm trap at 37 °C for 16 h. The Stage L3 *C. elegans* were placed directly onto the biofilm trap. A noxious blue light source from the epifluorescence microscope (Nikon, Japan) was then used to illuminate the biofilm spot and stimulate the repulsion of *C. elegans*. The

duration of *C. elegans* escaping from the biofilm spot was recorded using a timer.

Food choice assay

As previously described [18], *P. aeruginosa* PAO1 and *E. coli* OP50 lawns (circular shape with diameter of 1.2 cm) were grown 2.5 cm apart from each other at 37 °C for 16 h. After being washed by PBS buffer twice, 60 nematodes at L3 stage were transferred in the middle between the lawns of *P. aeruginosa* PAO1 and *E. coli* OP50. The nematodes were incubated at room temperature, allowing the nematodes to choose their preference between both species. The number of nematodes on either bacterial lawn was enumerated at 2, 4, 6, 10, and 24 h. The choice index was tabulated as follows: (worm number in PAO1 colonies − worm number in OP50 colonies)/total worm number used.

Biofilm protection assay

The overnight cultures of *P. aeruginosa* strains and *E. coli* OP50 were inoculated and grown in 2 ml LB medium at 37 °C for 16 h with shaking at 200 rpm. The bacterial culture was centrifuged to collect the cell pellet. The cell pellet was resuspended with 50 µl 0.9% NaCl. For *P. aeruginosa* strains, their cultures were then transferred to the center of the NGM plate to make a concentrated spot (diameter ~3 mm). On the same plate, OP50 *E. coli* was transferred and spread with a spreader over the remaining parts of the agar, leaving a 2 mm gap between the *P. aeruginosa* spot and the OP50 lawn. The NGM plate was incubated for 16 h at 37 °C to allow the growth of *P. aeruginosa* biofilm, which represented the "tougher" biofilm trap and OP50 lawn the susceptible biofilm.

On each plate, five individual Stage L3 *C. elegans* were transferred and placed onto the *P. aeruginosa* biofilm spot. Alternatively, as controls, *E. coli* OP50 treated with exogenously added 0 or 1 µg ml^{-1} Pel, Psl were used as biofilm spots. The extent of roaming by the *C. elegans* on the OP50 lawn after leaving the biofilm spot was quantified by counting the 5 × 5 mm grid squares being covered by *C. elegans* tracks every 10 min till the 60th minute. At least five replicate plates from three independent trials were conducted for the experiment.

Reproduction assay of *C. elegans* on bacterial lawns

To observe if the five nematodes could reproduce and expand their population after leaving the *P. aeruginosa* biofilm trap and reaching the OP50 lawn, the plates from the biofilm protection assay were further incubated at room temperature for 24 h to allow the emergence of L1 *C. elegans* progenies. Only the L1 *C. elegans* progenies on the OP50 lawns were enumerated with a tally counter.

Alternatively, when *E. coli* OP50 treated with 1 µg ml^{-1} Pel or Psl were used as biofilm spots, the plates from the *E. coli* biofilm protection assay were further incubated at room temperature for 120 h to allow the emergence of L1 *C. elegans* progenies. Only the L1 *C. elegans* progenies on the OP50 lawns were enumerated every 24 h with a tally counter. At least five replicate plates from three independent trials were conducted for the experiment.

Exopolysaccharide extraction

As previously described [19], Pel, Psl, and alginate were extracted by growing Δ*pslBCD*/p$_{lac}$-*YedQ*, Δ*pelA*/p$_{lac}$-*YedQ*, and Δ*mucA*Δ*pelA*Δ*pslBCD* static biofilms respectively on standard Petri dishes containing 15 ml LB supplemented with appropriate antibiotics at 37 °C for 16 h. The biofilms were collected and separated from the supernatant by centrifugation at 10,000 *g* for 5 min. The cell pellet was resuspended in 0.9% NaCl and treated with mild water-bath sonication (Elmasonic P120H, power = 50%, frequency = 37 KHz, 5 min) to separate the cells from the surface-associated matrix. The cells were then separated from the matrix by centrifugation, leaving behind the crude matrix extract.

The crude extract was then further treated by removal of eDNA by precipitation with 25% ethanol and 0.1 M $CaCl_2$. Extracellular proteins were then removed from the extract with 0.5 mg ml^{-1} proteinase K at 60 °C for 1 h and inactivation at 80 °C for 30 min. The extract was then filtered with centrifugal filter (<3 kDa) to remove the metabolites. The extract was then lyophilized and resuspended in sterile ddH_2O.

Exogenous addition of exopolysaccharides to non-polysaccharide-producing strains

The Δ*wspF*Δ*pelA*Δ*pslBCD* and Δ*mucA*Δ*algT* cells from growth culture (described in the previous section on growth conditions) were washed with 0.9% NaCl and centrifuged at 13,000 × *g* for 3 min. Varying concentrations (0, 0.5, 1, and 2 µg ml^{-1}) of Pel or Psl were mixed and resuspended in the Δ*wspF*Δ*pelA*Δ*pslBCD* cell pellet, while similar procedures were used for alginate and the Δ*mucA*Δ*algT* cell pellet. To test the effects of polysaccharide addition to a different bacterial species, *E. coli* OP50 was treated with 0 or 1 µg ml^{-1} Pel, Psl, or alginate.

The 30 µl of bacteria + exopolysaccharide mixture was transferred to NGM petri dish (3 cm diameter) for spreading with a spreader. The culture was incubated overnight to allow biofilm growth. At least 50 individuals of Stage L3 *C. elegans* were transferred from the feeding plate to the

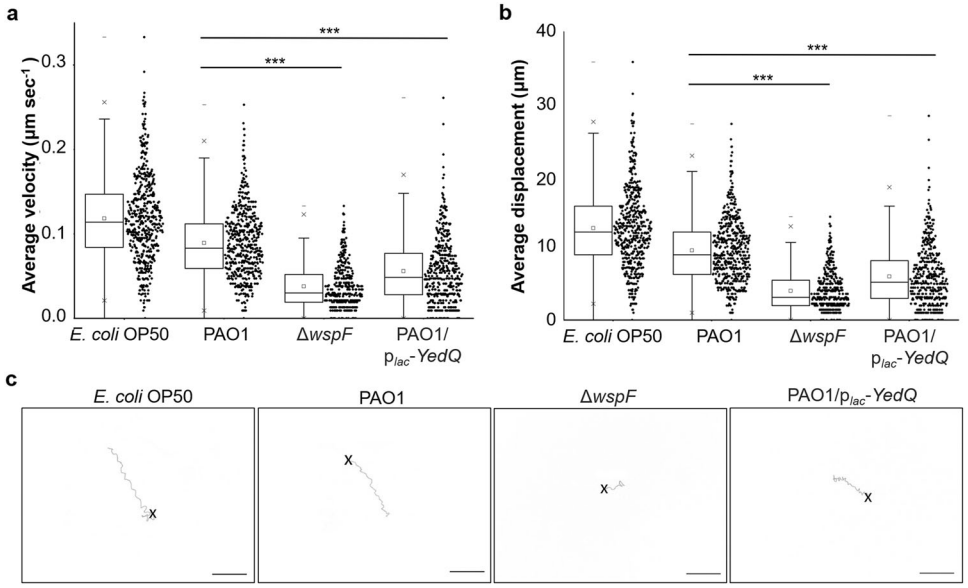

Fig. 1 Biofilms impede locomotion and restrict roaming of *C. elegans*. **a** Average velocity, **b** average displacement, and **c** representative tracks traveled by *C. elegans* ($N \geq 150$) on OP50, PAO1, Δ*wspF*, and PAO1/p_{lac}-*YedQ* lawns. Means and SD are shown. ***$P < 0.001$, one-way ANOVA.

center of the biofilm with a titanium wire picker. The nematodes were given 5 min for them to adapt to the fresh environment. Before recording the video of each nematode, the plate was tapped for three to four times to stimulate the *C. elegans* roaming in the bacterial biofilm lawn.

Prevalence of Pel and Psl genes in microbial species

The IMG portal [20, 21] was used to search for sequenced microbial species containing Pel and Psl synthesis genes using (date accessed: 22 June 2020). The identified microbial species (*P. aeruginosa*, other *Pseudomonads*, and non-*Pseudomonas* species) were enumerated and tabulated as percentage of total species.

Prevalence of mutated WspF protein in sequenced *P. aeruginosa* strains

The WspF protein sequence of prototypic PAO1 was aligned to that from all sequenced *P. aeruginosa* genomes via the DIAMOND BLASTP tool [22] (date accessed: 22 June 2020). Mismatches with one to six amino acids were listed, with 750 *P. aeruginosa* sequenced genomes being identified.

Results

Biofilms impede locomotion and restrict roaming of *C. elegans*

As a proof-of-concept showing that biofilms can impede *C. elegans* and restrict its roaming behavior, we first need to constitutively elevate c-di-GMP signaling and promote biofilm formation via the *wsp* operon, which controlled both *pel* and *psl* transcription [10]. We employed the Δ*wspF* mutant whose mutation of *wspF* repressor causes the derepression of WspR DGC, resulting in increased biofilm formation via the production of Pel and Psl [10] (Supplementary Fig. S1a). It is important to note that Δ*wspF* was frequently isolated in in vitro and in vivo biofilm infections [23, 24]. By aligning the WspF protein sequence in prototypic PAO1 to all sequenced *P. aeruginosa* isolates, we also found 750 isolates with one to seven mismatches in the WspF protein (Supplementary Data S1), indicating the prevalence in *wspF* mutations in nature. Furthermore, to confirm that our observations was due to c-di-GMP signaling per se and not attributed to possible pleiotropic effects of the *wsp* operon, we also expressed the p_{lac}-*YedQ* plasmid containing an *E. coli YedQ* DGC in wild-type PAO1 to constitutively elevate intracellular c-di-GMP levels and boost Pel and Psl production [24]. While cell number in the biofilm mainly remained consistent across the wild type and mutants, both Δ*wspF* and PAO1/p_{lac}-*YedQ* produced significantly higher exopolysaccharides than PAO1 wild type (Supplementary Fig. S2).

Interestingly, as compared to *E. coli* OP50 (Supplementary Video 1) and wild-type PAO1 (Supplementary Video 2), we observed that the nematodes moved at a slower pace and were easily trapped in the aggregates formed by Δ*wspF* mutant (Supplementary Video 3). By tracking the nematodes, we showed a significant reduction in velocity (Fig. 1a) and displacement (Fig. 1b) undertaken by the Δ*wspF* mutant as compared to wild-type PAO1. The nematodes moved constantly, albeit at lower velocity, on Δ*wspF* biofilms, indicating that they did not stop to rest or adopt punctuated (stop-go) movements (Supplementary Fig. S3). Higher biofilm formation by the Δ*wspF* mutant

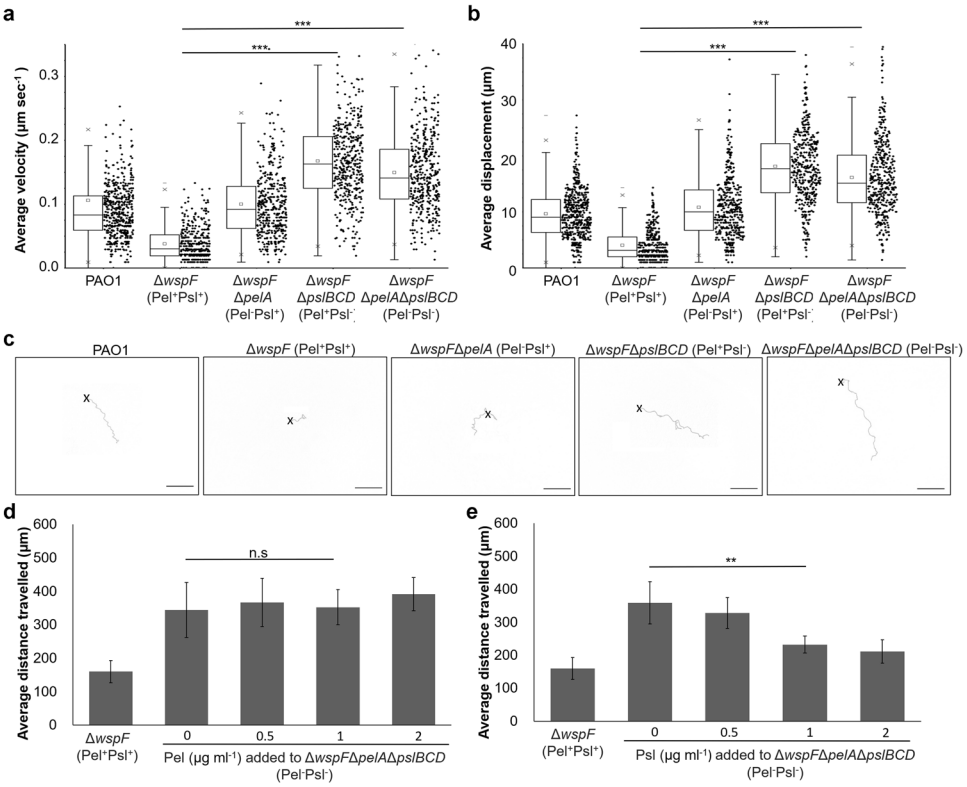

Fig. 2 Psl is more important than Pel at impeding nematode locomotion under influence by *wsp* operon. **a** Average velocity, **b** average displacement, and **c** representative tracks traveled by *C. elegans* ($N \geq 150$) on EPS mutant lawns. Changes to average distance traveled by *C. elegans* after exogenous addition of **d** Pel or **e** Psl to $Pel^{-}Psl^{-}$ strain. Means and SD are shown. $^{**}P < 0.01$, $^{***}P < 0.001$, ns not significant, one-way ANOVA.

also significantly restricted the roaming ability of *C. elegans* to explore the plate, which was reflected by highly localized tracks (Fig. 1c) undertaken by *C. elegans* on Δ*wspF* lawn. Upon closer inspection of the tracks, we also observed that the nematodes switched between forward and reverse locomotion frequently on Δ*wspF* lawn, as compared to that of wild-type PAO1 lawn where the nematode moved in a linear direction (Fig. 1c).

Similarly, the PAO1/p_{lac}-*YedQ* with increased biofilm formation could also dampen nematode motility and its ability to roam (Fig. 1a–d and Supplementary Video 4), thus corroborating with the results from *wsp* operon. This showed that the biofilms could cause the quagmire phenotype for *C. elegans*.

Psl is more important than Pel at impeding nematode locomotion under influence by *wsp* operon

Since the *wsp* operon controls both Pel and Psl production [10], we next asked which exopolysaccharide played a larger role in the quagmire phenotype. To maximize the phenotypic effects of the EPS in question and ensure that our results were solely dependent on one EPS, we mutated the EPS genes in the Δ*wspF* mutant. The presence of *wspF* mutation would boost the production of the exopolysaccharide whose synthesis genes were not mutated. Hence, in this paper, unless specified otherwise, the $Pel^{+}Psl^{+}$ referred to Δ*wspF*; $Pel^{+}Psl^{-}$ was Δ*wspF*Δ*pslBCD*; $Pel^{-}Psl^{+}$ was Δ*wspF*Δ*pelA*; $Pel^{-}Psl^{-}$ was Δ*wspF*Δ*pelA*Δ*pslBCD*.

We found that the loss of Pel and Psl in $Pel^{-}Psl^{-}$ completely abolished the quagmire phenotype, allowing the nematode to move at normal velocity and roam the bacterial lawn easily (Figs. 2a–c and S4 and Supplementary Video 5). Surprisingly, loss of Pel in $Pel^{-}Psl^{+}$ did not completely abolish the quagmire phenotype, while Psl loss in $Pel^{+}Psl^{-}$ was comparable to $Pel^{-}Psl^{-}$ (Figs. 2a–c and S4 and Supplementary Videos 6 and 7). While both exopolysaccharides were involved in the quagmire phenotype, Psl played a more important role in the quagmire phenotype as compared to Pel.

In the similar fashion, we inserted the p_{lac}-*YedQ* plasmid into the EPS mutants and ultimately found that these results corroborated with our observations on the *wsp* operon (Supplementary Fig. S5 and Supplementary Videos 4 and 8–10). Nevertheless, we found that our results were mainly dependent on the presence of the exopolysaccharide in the biofilm matrix, as we found qualitatively identical results when we used non-*wspF*-mutated backgrounds on PAO1, Δ*pelA*, Δ*pslBCD*, and Δ*pelA*Δ*pslBCD* mutants (Supplementary Fig. S6 and Supplementary Videos 2 and 11–13).

To confirm that the physical presence of the exopolysaccharide could contribute to the quagmire phenotype, we added Pel or Psl extracts exogenously to the $Pel^{-}Psl^{-}$ strain at increasing concentrations. While increasing concentrations of Pel did not establish the quagmire phenotype in the $Pel^{-}Psl^{-}$

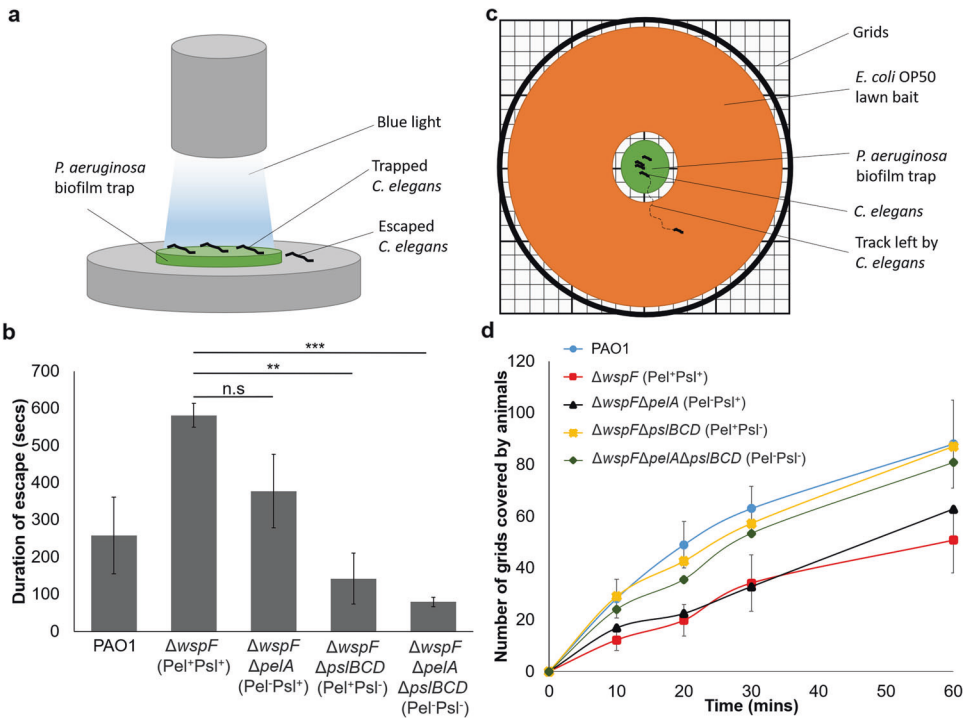

Fig. 3 Psl immobilizes and delays *C. elegans* from escape and attacking susceptible biofilms. a Schematic representation of trapping *C. elegans* by biofilms under blue light repulsion. **b** Duration taken by *C. elegans* to escape biofilm trap. **c** Schematic representation of trapping and baiting *C. elegans*. **d** Extent of roaming of *C. elegans* on bait biofilm after leaving the trap biofilm. Means and SD from triplicate experiments are shown. $^{**}P < 0.01$, $^{***}P < 0.001$, ns not significant, one-way ANOVA.

(Fig. 2d), we found that more than 1 μg ml^{-1} Psl could establish the quagmire phenotype, to the point of being similar to Δ*wspF* (Fig. 2e). This also corroborated with the levels of exopolysaccharide extracted from the biofilms (Supplementary Fig. S2b). Hence, addition of physiologically relevant Psl concentrations to the bacterial cells could alter the biofilm matrix and impede nematode motility.

Psl immobilizes and delays *C. elegans* from grazing susceptible biofilms

Since *C. elegans* possesses chemotaxis behavior like most animals, where it moves in response either from repellents (such as noxious repellents and predators) or to attractants (such as prey, mate, and odorants) [25], we next examined the implications of Psl-mediated interference on *C. elegans* motility.

Using a modified repulsion assay [26], we designed an escape assay where we first placed *C. elegans* in the biofilm "trap" and shone a direct beam of blue light on the biofilms (Fig. 3a). Blue light was previously shown to be a noxious yet harmless repellent under brief exposure [26], so this would encourage *C. elegans* to leave the biofilm as soon as possible. By observing the average duration required by the nematodes to escape the biofilm "trap" into the safe zone, we observed that the *C. elegans* took significantly longer time to escape from the Pel$^+$Psl$^+$ and Pel$^-$Psl$^+$ mutants, with some of them even unable to escape from the biofilm "trap" within 10 min (Fig. 3b). In contrast, it took lesser time for the animals to escape from the Pel$^-$Psl$^-$ and Pel$^+$Psl$^-$ biofilms (Fig. 3b). This showed that Psl could not only impede *C. elegans* motility, it could effectively help the biofilm immobilize *C. elegans*.

We next asked what could be the possible benefit brought to the biofilms by immobilizing a predatory *C. elegans* within themselves. Since *C. elegans* is a predator which roamed around in search for food, we tested if Psl-producing biofilms could prevent or delay the roaming of *C. elegans*, so that the non-Psl-producing biofilms or susceptible biofilms could be spared from nematode predation. Since our food preference assay had shown that *C. elegans* prefer *E. coli* OP50 over *P. aeruginosa* PAO1 (Supplementary Fig. S7a), we designed an assay which set the *P. aeruginosa* biofilm as nematode "trap" in the center of the petri dish, which was surrounded by the susceptible *E. coli* OP50 biofilm as "food bait" to motivate the nematodes to leave the trap (Fig. 3c). We then compared the extent of nematode tracks on the bait after their escape from the *P. aeruginosa* trap. Worms notably moved across a larger area of the bait over time after escaping quickly from the poorer trap (Pel$^-$Psl$^-$ and Pel$^+$Psl$^-$ mutants), whereas better traps (Pel$^+$Psl$^+$ and Pel$^-$Psl$^+$ mutants) could either reduce or completely prevent *C. elegans* from grazing on the bait, by delaying exit or immobilizing the nematodes (Fig. 3d).

This finding had significant implications on the recolonization ability of *C. elegans* on the susceptible OP50 lawns, where nearly 50% fewer L1 progenies ($P < 0.01$) were observed growing on OP50 lawns after the adult nematodes had escaped from better traps (Pel$^+$Psl$^+$ and Pel$^-$Psl$^+$ mutants) and recolonized on susceptible OP50 lawns

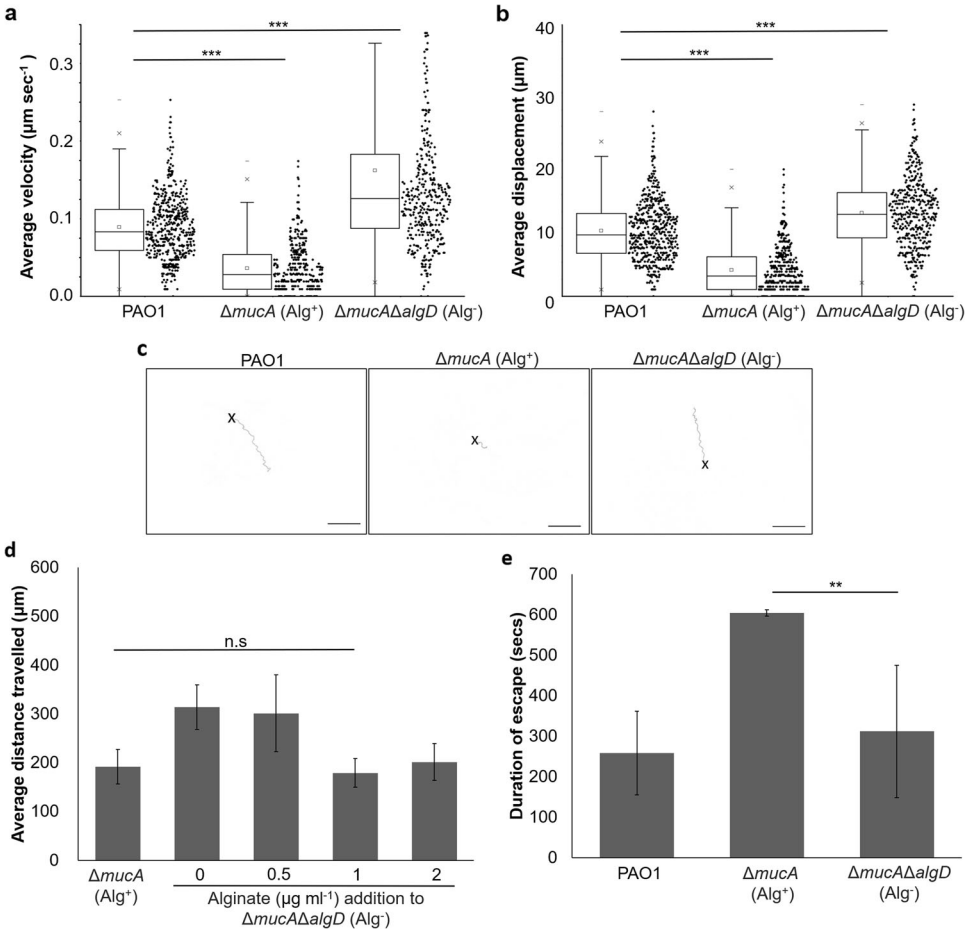

Fig. 4 Role of alginate in the quagmire phenotype. a Average velocity, **b** average displacement, and **c** representative tracks travelled by *C. elegans* ($N \geq 150$) on PAO1, Alg^+, and Alg^- lawns. **d** Exogenous addition of alginate to Alg^- strain. **e** Duration taken by *C. elegans* to escape biofilm trap. Means and SD are shown. $^{**}P < 0.01$, $^{***}P < 0.001$, ns not significant, one-way ANOVA.

(Supplementary Fig. S7b). Hence, Psl was comparatively more important than Pel in entrapping predators to delay or prevent susceptible biofilms from predation, thereby improving the overall survival of the biofilms.

Role of other matrix components in the quagmire phenotype

Other than Pel and Psl, the *P. aeruginosa* biofilm matrix comprises of a multitude of matrix components, each of which might play a role in dictating how *C. elegans* move across the biofilms. Since CdrA adhesion protein and eDNA were not significantly involved in the quagmire phenotype (Supplementary Fig. S8), we tested alginate, the third and final exopolysaccharide in *P. aeruginosa*. Alginate production was controlled by the *muc* c-di-GMP-signaling operon, and was responsible for mucoidy of a subset of clinical isolates in cystic fibrosis patients [27, 28]. Since its production was inversely regulated with Pel/Psl exopolysaccharides production, the presence of Pel and Psl is minimal in mucoid strains [29]. We employed the Δ*mucA* mutant (Alg^+) whose mutation of *mucA* repressor causes the derepression of MucR DGC, resulting in increased mucoid biofilm formation via the production of alginate (Supplementary Fig. S1b). With higher exopolysaccharide concentration in Alg^+ strain than PAO1 wild type (Supplementary Fig. S2b), we showed that increased production of alginate could also impede *C. elegans* motility (Fig. 4 and Supplementary Video 14). Mutagenesis of alginate synthesis gene *algT* in the Δ*mucA*Δ*algT* mutant (Alg^-) resulted in abrogation of quagmire phenotype, allowing the *C. elegans* to roam the bacterial lawns freely at normal speed (Figs. 4a–c and Supplementary Fig. S9 and Supplementary Video 15). Exogenous addition of alginate to the Alg^- mutant deficient in alginate production can restore the quagmire phenotype at 1 μg ml^{-1} (Fig. 4d). Similar to Psl, alginate could also immobilize *C. elegans* and prevent its escape from noxious blue light (Fig. 4e). This implied that under the influence of *muc* operon, alginate was solely important to impeding nematode locomotion.

Discussion

Bacteria are often the target of predation by bacterivores, such as nematodes and amoebae in the environment, and

phagocytes in the human body. To ensure their survival, bacteria produce a plethora of virulence factors that can effectively kill their predators, such as phenazines, hydrogen cyanide, and T3SS [30–32]. Such virulence factors typically require hours to days to kill *C. elegans*, which are typically demonstrated in fast paralytic (8–24 h) and slow killing assays (days to weeks) [33].

Bacteria also form biofilms whose exopolymeric matrix offers protection to bacteria by resisting predation. To our knowledge, we showed a hitherto unreported function of the biofilm matrix, which was to impede nematode locomotion and alter its grazing ability. In the case of *P. aeruginosa*, Psl and alginate exopolysaccharides were important in entrapping and restricting nematode movement, thereby hampering *C. elegans*' ability to roam and forage for food. This observation was applicable to *C. elegans* of various ages and sizes tested, where young L1 larvae and adults had retarded motility on *P. aeruginosa* biofilms containing Psl or alginate, with increased propensity to be immobilized on the biofilm traps (Supplementary Fig. S10). Furthermore, this retarded motility was observed to be consistent for longer periods (up to 1 h), implying the long-lasting effect of biofilms on nematode motility (Supplementary Fig. S11).

While Pel and Psl genes were commonly found in most environmental and clinical isolates, alginate was only expressed in a subset of mucoid clinical isolates [34], thus Psl could be prevalently employed by *P. aeruginosa* biofilms in nature as compared to alginate. Nevertheless, the redundancy of exopolysaccharides involved in the quagmire phenotype conferred versatility in *P. aeruginosa* to respond to specific stimuli and adjust the composition of its biofilm matrix accordingly. This improved the survival of *P. aeruginosa* biofilms in face of varying stresses and predators.

While Pel is involved in scaffolding with eDNA-crossing-linking properties [35, 36], its viscosity allows bacterial cells to spread within the biofilm matrix, which may explain why the Pel exopolysaccharide was significantly less effective at impeding nematode locomotion than Psl [37].

Yet for Psl, it is a rigid polymer which increases effective cross-linking of cells in the biofilm matrix, thus strengthening the scaffold and promoting the formation of stiff microcolonies [37]. Without Psl, the biofilm matrix becomes less rigid [37], which may indicate why *C. elegans* can move across Psl-deficient biofilms easily. Nonetheless, further investigation of how these "sticky" components interact with the proteinaceous outer cuticle of *C. elegans* is warranted.

As for eDNA and CdrA adhesion protein, they do not have an observable effect on locomotion, which can be attributed to their lower presence in the matrix composition as compared to exopolysaccharides [38]. While biofilm matrix is directly involved in impeding nematode motility, upstream c-di-GMP-signaling proteins can indirectly influence the quagmire phenotype. Clearly, biofilms formed by DGC mutants (Δ*wspR* and Δ*mucR*) are worse off than wild-type PAO1 in retarding *C. elegans* locomotion (Supplementary Fig. S12), emphasizing their importance in the biofilms' quagmire phenotype.

Our findings have several implications in nature and clinical settings. In the environment, the biofilms could impede the mobility of nematode as a form of protection from grazing and act as trap to reduce further damage to susceptible biofilms, thereby improving the overall survival of the biofilms. With diverse soil microbial species that are known to interact with nematodes [39], *C. elegans* remain susceptible to a variety of pathogens, such as *Leucobacter* and *Corynebacteria* species, which could form robust biofilms that interact with *C. elegans* [40, 41]. Furthermore, certain *Leucobacter* strains could form aggregates, which cause swimming worms to stick together by their tails in a dead-inducing entrapment (star formation) [42]. This adds a layer of complexity in predator–prey interactions for future work on other bacterial species and bacterivores. Our findings can be also used as a gauge to test the physical parameters of various components in the biofilm matrix against predation.

Our results also raised an interesting question into the prevalence of Pel and Psl synthesis genes possessed by different microbial species. A search of sequenced bacterial genomes from the *C. elegans* native microbiota [43, 44] for Pel and Psl synthesis genes using the IMG portal revealed that other *Pseudomonas* species, such as *Pseudomonas protegens* and *Pseudomonas lurida*, and non-*Pseudomonas* species such as *Delftia acidovorans* contained Pel genes (Supplementary Data S2). One the other hand, Psl genes were exclusively found in *Pseudomonas* species (Supplementary Data S2). This raised the possibility that *C. elegans* could encounter such biofilms in the soil.

When we expanded the search to all sequenced microbial species, it was noted that more *Pseudomonas* species, such as *Pseudomonas protegens*, *Pseudomonas fluorescens*, and *Pseudomonas chlororaphis*, and non-*Pseudomonas* species, such as *Burkholderia*, *Paraburkholderia*, and *Pseudoalteromonas*, contained Pel genes (Supplementary Fig. S13a and Supplementary Data S3). Yet, Psl genes remained almost exclusive to *P. aeruginosa* and other *Pseudomonas* species (Supplementary Fig. S13b and Supplementary Data S3). Such microbial species were isolated from a variety of locations, ranging from clinics to soil, plant roots, and aquatic settings (Supplementary Data S3), indicating the prevalence of such biofilm exopolysaccharides in the environment where *C. elegans* could encounter. Furthermore, we observed that the addition of Psl or alginate to another species, *E. coli* OP50, could also impede nematode motility and even reproduction rate of its predator

(Supplementary Fig. S14), raising the possibility that non-Psl-producing microbial species could utilize Psl or inhabit with Psl-producing species for similar purposes.

In clinical settings, clinical isolates such as mucoid strains with induced alginate expression and rough small colony variant strains with overexpression of Psl are frequently isolated from patients with cystic fibrosis, where phagocytosis of biofilm cells by immune cells was severely hampered [45, 46]. Our findings that the biofilm exopolysaccharides could impede predator motility raise the possibility that leukocytes have reduced migration and motility across the biofilms in human infections, thereby preventing biofilm clearance by the immune system. This warrants the need for development of anti-biofilm agents specific against biofilm matrix components [47].

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Author contributions SLC designed methods and experiments. SYC, SYL, and ZS carried out laboratory experiments, analyzed the data, and interpreted the results. SLC, SYL, and SYC wrote the paper. All authors have contributed to, seen, and approved the paper.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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