

# Biofilm matrix cloaks bacterial quorum sensing chemoattractants from predator detection

## Authors and Affiliations:

Shaoyang Li <sup>1a</sup>, Sylvia Yang Liu <sup>1a</sup>, Shepherd Yuen Chan <sup>1a</sup>, Song Lin Chua <sup>1, 2, 3, 4 \*</sup>

1. Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic

University, Kowloon, Hong Kong SAR China

2. State Key Laboratory of Chemical Biology and Drug Discovery, The Hong Kong

Polytechnic University, Kowloon, Hong Kong SAR China

3. Shenzhen Key Laboratory of Food Biological Safety Control, The Hong Kong

Polytechnic University, Kowloon, Hong Kong SAR China

4. Research Centre for Deep Space Explorations (RCDSE), The Hong Kong Polytechnic

University, Kowloon, Hong Kong SAR China

<sup>a</sup>Co-first authors

\*corresponding author:

Song Lin Chua

[song-lin.chua@polyu.edu.hk](mailto:song-lin.chua@polyu.edu.hk)

**Abstract:**

Microbes often secrete high levels of quorum sensing (QS) autoinducers into the environment to coordinate gene expression and biofilm formation, but risk detection and subsequent predation by bacterivorous predators. With such prominent signalling molecules acting as chemoattractants that diffuse into the environment at alarmingly high concentrations, it is unclear if bacterial cells can mask their chemical trails from predator detection. Here, we describe a microbial-based anti-detection adaptation, termed as ‘biofilm cloak’, where the biofilm prey produced biofilm matrix exopolysaccharides that ‘locked’ and reduced the leaching of autoinducers into the milieu, thereby concealing their trails to the detection by the bacterivorous *Caenorhabditis elegans* nematode. The exopolysaccharides act as common good for the non-producers to hide their autoinducers from predator detection. Deficiency in chemosensory gene *odr-10* in mutant animals abrogated their ability to detect autoinducers and migrate towards their prey in a directed manner, which led to lower population growth rate of animals. Hence, restriction of bacterial communication activities to the confinements of biofilms is a novel approach for predator evasion, which plays a fundamental role in shaping ecological dynamics of microbial communities and predator-prey interactions.

**Keywords:** Biofilm, *Pseudomonas aeruginosa*; *Caenorhabditis elegans*; c-di-GMP; biofilm matrix

## Introduction:

Anti-predator adaptations are mechanisms that assist the prey organisms in their struggle for survival against predators, where many are evasive in nature, such as camouflage and masquerade. In this pursuit, experimental model systems using microbes or higher organisms, have served as an important bridge between nature's complexity and simplified theoretical studies (1, 2). We and others had recently shown novel predator-prey interactions using the bacteria and nematode, *Caenorhabditis elegans* as model systems (3, 4), thus revealing novel insights into predator-prey relationships in the microbial world (5). Predators such as *C. elegans*, can detect the attractive odorants released by bacteria, such as isoamyl alcohol and diacetyl, and locate their prey (6, 7). *C. elegans* depends on its olfactory system to detect bacterial prey, where neuron pairs present in its head and tail are important for detection of chemical cues in the environment (8).

Bacteria spend nearly their entire lives in biofilms, which are multicellular communities encased in self-produced exopolymeric matrix. To coordinate biofilm formation, bacteria employ quorum sensing (QS) to crosstalk among themselves via the release of QS autoinducers. One classical example is *Pseudomonas aeruginosa*, which employs both homoserine-based (*las* and *rhl*) and quinolone-based (*pqs* and *iqs*) QS systems for intercellular communication and subsequent biofilm formation. However, autoinducers could be detected easily by predators and immune cells (9, 10), thus posing a dilemma for microbes in choosing between biofilm formation and predator evasion.

Here, we showed that the biofilm matrix acted as cloak to hide QS signals and prevent predator detection. Specifically, exopolysaccharides released by *P. aeruginosa* biofilms was crucial in 'locking' *N*-butanoyl-L-homoserine lactone (BHLs) within the biofilms to reduce their leaching into the environment, thereby preventing detection by *C. elegans*. The 'biofilm cloaking' feature was a common good for non-exopolysaccharide producers and other bacterial species.

Correspondingly, *odr-10*, a *C. elegans* chemosensory gene, was crucial in the detection of autoinducers released from biofilms. Hence, our work demonstrated a hitherto described biofilm cloaking phenotype which plays a fundamental role in shaping ecological dynamics of microbial communities and predator-prey interactions.

## Results:

### *C. elegans* cannot detect exopolysaccharide-producing biofilms.

We first tested if *C. elegans* would prefer the bacteria deficient in biofilm matrix production over wild-type bacteria in a typical choice assay. Since *P. aeruginosa* mainly produce Pel and Psl exopolysaccharides in its biofilms (11), we evaluated the conventional choice assay which allowed the animals to choose between exopolysaccharide deficient ( $\Delta pelA$ ,  $\Delta pslBCD$  or  $\Delta pelA\Delta pslBCD$ ) biofilms and PAO1 wild-type biofilm. We found that the exopolysaccharide mutant biofilms were preferred by the animals than wild-type PAO1 biofilm (**Supplementary Figure S1**). As proof-of-concept showing if *C. elegans* can detect the metabolites released by the biofilms into the environment, we employed a modified assay of chemotaxis, where secreted biofilm metabolites were used as chemoattractant for *C. elegans* detection (**Figure 1a**). Briefly, we first grew *P. aeruginosa* biofilms on 0.2- $\mu$ m filter overlaid NGM agar at opposite ends of the petri dish for 24 hrs, allowing the secreted metabolites to seep into the agar. We then removed the filter (with biofilms attached) from the agar to eliminate the possibility that *C. elegans* could detect bacterial biofilm cells directly, followed by placement of *C. elegans* in the middle of agar plate. This enabled the *C. elegans* to detect the presence of pure biofilm-secreted metabolites which had leached into the agar plate.

In this case, *C. elegans* were given the choice to choose between the metabolites secreted by wild-type PAO1 and the mutant deficient in producing a biofilm matrix component, namely  $\Delta pelA$ ,  $\Delta pslBCD$  and  $\Delta pelA\Delta pslBCD$  in our modified chemotaxis assay. The animals preferred the metabolites secreted by  $\Delta pelA$ ,  $\Delta pslBCD$  and  $\Delta pelA\Delta pslBCD$  biofilms over those from wild-type PAO1 control (**Figure 1b**), implying that both Pel and Psl were important in preventing the detection by *C. elegans*. We eliminated the possibility that different bacterial numbers could lead to differences in metabolite released, as CFU in biofilms remained nearly constant for PAO1 wild-type and its mutants (**Supplementary Figure S2**).

We further ascertained our results by boosting biofilm formation in *P. aeruginosa* via the induction of c-di-GMP signaling. C-di-GMP is a bacterial secondary messenger whose synthesis by diguanylate cyclases led to motility loss and biofilm formation, while breakdown

by phosphodiesterases led to biofilm dispersal (12-14). We employed the  $\Delta wspF$  mutant which promoted the constitutive expression of *wspR* DGC, resulting in increased biofilm formation via the production of Pel and Psl (15). Mutations in the *wspF* gene were frequently identified in biofilm-mediated *P. aeruginosa* infections (16, 17). By establishing a chemotaxis assay between wild-type PAO1 and pro-biofilm-forming  $\Delta wspF$ , we showed that the animals were highly attracted towards the region previously colonized by PAO1 biofilms instead of  $\Delta wspF$  biofilms, indicating the absence of a certain metabolite released by  $\Delta wspF$  biofilms (**Figure 1c**).

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  $\Delta wspF$  mutant. The presence of *wspF* mutation will boost the production of the exopolysaccharide. However, the loss of Pel or Psl in  $\Delta wspF\Delta pelA$ ,  $\Delta wspF\Delta pslBCD$  led to chemoattraction by *C. elegans* when placed in a chemotaxis assay with  $\Delta wspF$  (**Figure 1d**). Loss of both Pel and Psl in  $\Delta wspF\Delta pelA\Delta pslBCD$  exacerbated the effect, where *C. elegans* were highly attracted to the mutant as compared to wild-type strain (**Figure 1d**).

To confirm that our observations was attributed to c-di-GMP signaling *per se* and not due to pleiotropic effects of the *wsp* operon, we first expressed a *plac-YedQ* plasmid containing a *YedQ* DGC in wild-type PAO1 to constitutively elevate intracellular c-di-GMP levels and boost Pel and Psl production (17). We found qualitatively identical results which corroborated with our observations using  $\Delta wspF$  (**Figure 1c, Supplementary Figure S3**). Although cell number in the biofilm remained consistent across the wild-type and mutants (**Supplementary Figure S2**), both  $\Delta wspF$  and PAO1/*plac-YedQ* were previously shown to produce significantly higher exopolysaccharides than PAO1 wild-type (3). We also found no significant differences concerning other mutants of known biofilm matrix components, such as adhesion proteins and eDNA, indicating that they are not involved in the ‘safehouse’ phenotype (**Supplementary Figure S4**). Moreover, other polysaccharides, such as cellulose and pectin, could not prevent the detection of BHL by *C. elegans* (**Supplementary Figure S4B**).

It is also crucial to find out if exogenous addition of Pel or Psl to a non-exopolysaccharide mutant ( $\Delta pelA\Delta pslBCD$ ) could prevent their colonized area from being detected by the predator. Since our previous study had shown that PAO1 wild-type biofilm contained an estimated of  $1 \mu\text{g ml}^{-1}$  Pel and Psl in the biofilm matrix, we added  $1 \mu\text{g ml}^{-1}$  Pel or Psl to the  $\Delta pelA\Delta pslBCD$  and grew a biofilm overnight before removing the biofilm from the agar. Interestingly, we found that *C. elegans* preferentially chose the secreted metabolites of  $\Delta pelA\Delta pslBCD$  control over  $\Delta pelA\Delta pslBCD + 1 \mu\text{g ml}^{-1}$  Pel or Psl (**Figure 1e**).

### ***C. elegans* detects BHL QS autoinducer in distance-dependent manner.**

We next asked what biofilm metabolite was detected by *C. elegans*. Since there were high levels of homoserine-based autoinducers in the biofilms, we hypothesized that the autoinducers could leach from biofilms into the environment, resulting in detection by any lurking predator. To test if *C. elegans* could detect the OdDHL and BHL leached from biofilms into the agar, we first placed pure OdDHL and BHL in the agar for seeping and diffusion across the agar, allowing the animals to detect the metabolites. We found that *C. elegans* could detect both OdDHL (C12-chain autoinducer for *las* QS) and BHL (C4-chain autoinducer for *rhl* QS) (**Figure 2a**), which corroborated with a previous finding that *C. elegans* could detect autoinducers (9). This could be attributed to the smaller molecular weight of BHL than OdDHL, where the concentration of BHL and OdDHL decreased over distance in the agar (**Figure 2b**). We tested other known metabolites (pqs, rhamnolipid, pyoverdine and pyocyanin) and secreted protein (lasB), where they were not detected by *C. elegans* (**Supplementary Figure 5**).

### **Exopolysaccharides accumulate BHL within biofilms.**

Since *C. elegans* could detect OdDHL and BHL in the agar, it is highly likely that our prior observations in Figure 1 could be attributed to wild-type PAO1 and mutant biofilms leaching different concentrations of OdDHL and BHL. We measured the concentration of BHL within the wild-type and mutant biofilms and found no significant differences among the biofilms (**Figure 2c**), indicating that there was no deficiency in BHL production in the biofilms. Interestingly, once outside the biofilm in the agar, there were higher concentrations of BHL in  $\Delta pelA$  and  $\Delta pelA\Delta pslBCD$  mutants, as compared to wild-type PAO1 and  $\Delta pslBCD$  (**Figure 2d**). We found qualitatively similar results for the exopolysaccharide mutants expressed with the

$\Delta wspF$  background, albeit at lower levels than those under the wild-type background (**Figure 2d**), indicating that overproduction of exopolysaccharides would prevent BHL from ‘leaking’ into the environment.

To confirm our observations that Pel exopolysaccharide could trap BHL and prevent leaching into the agar, we tested if *C. elegans* could detect the autoinducers leached in the agar after using purified Pel and Psl to trap the autoinducers on the filter. As control, purified Pel and Psl themselves were neither attractive nor aversive cues to the animals (**Figure S6**). We found that both purified exopolysaccharides at increasing concentrations could trap BHL and reduce BHL entry onto the agar, thereby preventing the detection by *C. elegans* (**Figure 2e**). This could be attributed to the ‘stickiness’ of the biofilm matrix which allowed it to trap small metabolites in a general manner (18). By breaking down the exopolysaccharides in the biofilm matrix with a known anti-biofilm glycosyl hydrolase, cellulase (19), we showed that cellulase-induced degradation of biofilm matrix resulted in the detection of BHL by the animals (**Figure 2f**), emphasizing the importance of biofilm matrix in ‘cloaking’ BHL from predators.

#### **Public good for non-producers and other microbial species**

Since most microbial communities in the environment are polymicrobial in nature, some microbial species are not producers of QS or exopolysaccharides. To address if non-QS producers and other microbial species could similarly be protected by the Pel exopolysaccharide from detection by *C. elegans* as a form of public good, we added different concentrations of Pel or Psl and AHL or BHL exogenously in various mixtures to a non-QS producer ( $\Delta lasI\Delta rhII$  mutant) and another bacterial species (*Escherichia coli* OP50).

For non-QS producer which could not produce AHL and BHL ( $\Delta lasI\Delta rhII$  mutant), *C. elegans* could not detect the  $\Delta lasI\Delta rhII$  mutant in the choice assay with PAO1 control (**Figure 3a**). Addition of pure AHL or BHL to  $\Delta lasI\Delta rhII$  mutant could restore the phenotype for *C. elegans* detection, but with the presence of purified Pel or Psl added to the biofilms, *C. elegans* could no longer detect the metabolites anymore (**Figure 3b**).

For another bacterial species (*E. coli* OP50) which produce its own QS molecules such as AI-2 (20), (**Figure 3c**). Exogenous addition of pure BHL to OP50 could restore the phenotype for *C. elegans* detection, but with the presence of purified Pel or Psl added to the biofilms, *C. elegans* could no longer detect the metabolites anymore (**Figure 3d**). This indicated that Pel and Psl could be used as a public good for bacteria to hide their ‘signatures’ from predator detection.

#### **Loss of function of *odr-10* gene in *C. elegans* eliminates biofilm prey detection.**

We next sought to examine how the autoinducer detection of biofilm prey by *C. elegans* are impacted when we limit the ability of *C. elegans* to detect autoinducers. We employed worms with a mutation in the *odr-10* gene, which causes loss of odorant detection to volatile compounds (21, 22). ODR-10 is a specific G-protein-coupled olfactory receptor that binds to diacetyl, which is a volatile odorant produced by various microbial species including lactic acid bacteria (7, 21, 22). We showed that the exploratory behavior of *odr-10* mutants was comparable to wild-type N2 animals (**Supplementary Figure S7**), but the mutant worms could not detect pure autoinducers (**Figure 4a**), even with Pel or Psl (**Supplementary Figure S8**), and differentiate between bacterial mutants (**Figure 4b**) in the choice assays.

Lastly, we examined if the loss of autoinducer detection by *C. elegans* had a significant impact on bacterial prey survival. After performing the choice assay, we fed the worms localised at their respective corners with fresh bacteria and enumerate the bacterial populations (**Figure 4c**). Since the animals preferred the metabolites secreted by  $\Delta pelA$ ,  $\Delta pslBCD$  and  $\Delta pelA\Delta pslBCD$  biofilms over those from wild-type PAO1 control, we observed the feeding-attributed reduction of  $\Delta pelA$ ,  $\Delta pslBCD$  and  $\Delta pelA\Delta pslBCD$  bacterial populations as compared to wild-type PAO1 control (**Figure 4d**). This indicated that the ability to sense autoinducers could result in the predator’s preference towards its prey, which had significant impact on bacterial prey survival.

## Discussion:

To ensure survival from bacterivorous predation, bacteria produce different types of virulence factors of protein or toxin in nature, which can effectively eliminate their predators (23-25). However, this requires hours to days in duration to kill *C. elegans*, as reflected by the quick paralytic killing and slow killing assays, respectively (26). Unable to react immediately to their rapidly changing plight, biofilms need to passively protect themselves from predator, where the biofilm matrix could either slow down the motility of predators to reduce predation (3), or act as barrier to prevent direct feeding.

However, given the plethora of proteins and metabolites being secreted into the environment by bacteria, it appeared to be inevitable that biofilms will attract predators. This is especially true when there are high concentrations of QS autoinducers secreted into the environment at high cell densities or in biofilms, where autoinducers were known chemoattractants to *C. elegans* (9, 10). This raised a longstanding fundamental question into how bacteria could avoid predator detection under such circumstances.

Here, we reveal a novel ‘stealth’ approach adopted by biofilms to ‘hide’ their biosignatures and prevent the release of QS autoinducers into the environment for predator detection (**Figure 5**). This was reflected in the previously undescribed role of biofilm exopolysaccharides, in the form of Pel and Psl, to trap BHL autoinducers within the biofilms and prevent them from leaching into the surrounding agar in a distance-dependent manner, thereby preventing *C. elegans* detection via ODR-10 olfactory receptor. However, the exact mechanisms of how ODR-10 mediates attraction of OdDHL and BHL remain unclear. Since ODR-10 could homo-oligomerise, and even form heteromers with STR-112, another related nematode receptor (27), we speculate that such complexity could possibly help in the detection of larger chemoattractants, including OdDHL and BHL. Similar to a police and criminal-like scenario (28), the criminals (bacterial cells) could conduct their illicit meeting (QS) in the safehouse (biofilms) and prevent leaks of their communication signals (autoinducers), where the police (*C. elegans*) could not hijack the signals for arrest (olfactory detection and predation).

249 Our findings also had implications in infectious diseases, where nearly 80% of bacterial  
250 infections were mediated by biofilms. While it is commonly known that biofilms could evade  
251 immune detection where its biofilm matrix acts as barrier against phagocytic clearance and  
252 detection by inflammatory factors (29), the exact mechanism of preventing detection by immune  
253 cells remain poorly understood. Although our lab-based approach used to study *C. elegans*  
254 detection of biofilm metabolites was artificial, our findings offered insight that the biofilms  
255 reduce the likelihood of immune detection by trapping its biosignatures in its matrix  
256 exopolysaccharides. Moreover, the biofilm matrix offers a multitude of functions in infections,  
257 with recently discovered examples of offering protection of tumor cells from anticancer  
258 chemotherapy and efficient pathogen transfer across food (30, 31), thereby complicating the role  
259 of biofilms in infections. Hence, use of antibiofilm agents to disrupt biofilms (32) could be a  
260 viable way of exposing the biofilm bacteria to immune detection and improve clearance by the  
261 host immune system.

## 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 manipulation, while *E. coli* OP50 was used for growth and maintenance of *C. elegans*. 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<sup>-1</sup> ampicillin and 15 µg ml<sup>-1</sup> gentamicin. For marker selection in *P. aeruginosa*, 30 µg ml<sup>-1</sup> gentamicin, 30 µg ml<sup>-1</sup> tetracycline or 200 µg ml<sup>-1</sup> carbenicillin were used, as appropriate.

### *Growth and maintenance of C. elegans*

As previously described (33), the bacterial lawn, such as *E. coli* OP50 and *P. aeruginosa* strains, were cultivated on nematode growth media (NGM) agar plates at 37 °C for 16 hrs. The laboratory *C. elegans* N2 nematode was transferred to the bacterial lawn on the nematode growth media (NGM) agar plates and cultivated at room temperature for 72 hrs for population expansion. The CX32 strain possessing the *odr-10* mutation was procured from the Caenorhabditis Genetics Center (CGC) for experiments.

### *C. elegans choice Assay of biofilm*

*P. aeruginosa* wild-type PAO1 and its respective mutants were first inoculated and grew in 2 ml of Luria-Bertani (LB) medium at 37 °C with shaking at 200 rpm for 16 hrs. After washing the overnight culture with 0.9% NaCl and adjustment of OD600 to 0.1, the 30 µl of bacterial cultures (control strain and test strain) were transferred directly to opposite ends of NGM petri dish. The cultures were spread carefully on the agar with a bacteriological spreader. The culture was incubated at 37 °C for 16 hrs to allow biofilm growth on the agar. At least 50 individuals of adult *C. elegans* from 3 independent trials were transferred from the feeding plate to the center of the petri dish with wire picker. Live animals localized at each biofilm spot were enumerated by 6 hrs.

### *C. elegans modified choice assay of biofilm metabolites*

*P. aeruginosa* wild-type PAO1 and its respective mutants were first inoculated and grew in 2 ml of Luria-Bertani (LB) medium at 37 °C with shaking at 200 rpm for 16 hrs. After washing the

overnight culture with 0.9% NaCl and adjustment of OD600 to 0.1, the 30 µl of bacterial cultures (control strain and test strain) were transferred to 0.2-µm filters (Nanopore) which were placed on opposite ends of NGM petri dish. The cultures were spread carefully on the filter with a bacteriological spreader, where the cultures should not be directly in touch with the NGM agar. The culture was incubated at 37 °C for 16 hrs to allow biofilm growth and diffusion of secreted metabolites through the filter into the NGM agar.

For chemoattractant test, the pure chemical (OdDHL, BHL, PQS, rhamnolipid, pyocyanin and pyoverdine) at 10 µM and purified polymers (pectin, cellulose and *P. aeruginosa* genomic DNA) at 10 µg ml<sup>-1</sup> was placed on the 0.2-µm filter one end of NGM plate, while its saline control (0.9% NaCl) was placed on the opposite end of the petri dish, followed by 16 hrs incubation at 37 °C.

The filters (containing biofilms or chemicals) on opposite ends of petri dish were carefully peeled off from the NGM agar, leaving behind the secreted biofilm metabolites in the agar. At least 50 individuals of adult *C. elegans* from 3 independent trials were transferred from the feeding plate to the center of the petri dish with wire picker. The animals were allowed to detect the secreted biofilm metabolites and migrate to the spot previously occupied by either control strain or test strain, where live animals localized at each spot were enumerated by 6 hrs.

#### *Tabulation of choice index (CI)*

The CI was tabulated in Equation 1:

$$(1) \frac{\text{Live animals on secreted metabolic spot left by test strain} - \text{Live animals on secreted metabolic spot left by control strain}}{\text{Total Live animals}}$$

CI>0.00 indicated animal preference for test strain or compounds, where CI<0.00 indicated animal preference for control strain or compounds. CI thresholds for non-preference were set as a range of -0.1 < CI < 0.1.

#### *Quantification of secreted autoinducers in biofilms and on agar*

As previously described (13), the biofilms grown on filter on agar were scrapped and transferred to 1 ml saline (0.9% NaCl). The biofilms were then homogenized to single cells using water sonication in ice bath for 15mins and 15-sec vortexing, to release the secreted autoinducers into

the solution. The cells were removed via centrifugation at 13000 g for 3 mins, and the supernatants containing autoinducers were filtered through 0.2- $\mu$ m filters.

As quantifying autoinducers in the agar, the agar was cut at different distances (0 cm, 1.5 cm and 3 cm) from the center of biofilm on filter, and transferred to 1 ml saline (0.9% NaCl). The agar was mashed into smaller pieces using a microtube pestle and placed at 4 °C overnight for metabolites to diffuse into the saline. The agar pieces were removed via centrifugation at 13000 g for 3 mins, and the supernatants containing autoinducers were filtered through 0.2- $\mu$ m filters.

Overnight culture of the reporter strain  $\Delta lasI\Delta rhII/p_{lasB}-gfp$  or  $\Delta lasI\Delta rhII/p_{rhIA}-gfp$  for quantifying OdDHL and BHL respectively, was adjusted to OD<sub>600</sub> = 0.1 using 2-fold ABTGC medium. 100  $\mu$ l of filtrate was added to 100  $\mu$ l of reporter strain in a 96-well plate (SPL, South Korea). As  $\Delta lasI\Delta rhII$  does not produce OdDHL and BHL,  $p_{lasB}-gfp$  or  $p_{rhIA}-gfp$  was induced by the addition of biofilm filtrates containing OdDHL or BHL respectively. GFP fluorescence from  $p_{rhIA}-gfp$  expression (expressed in relative fluorescence units, RFU) was measured for each well using a microplate reader (Tecan Infinite 2000) and was normalized to the OD<sub>600</sub> of each well. To quantify autoinducer concentration, the standard curves for OdDHL and BHL (using 0, 0.1, 1, 5, 10, 20 and 30  $\mu$ M) was generated using the similar assay for comparison to test samples. Experiments were performed in triplicate, and results are shown as the mean  $\pm$  s.d.

#### *Colony-forming unit (CFU) assay*

As previously described (34), the biofilms were quantified using the CFU assay. The biofilms grown on filter on agar were scrapped and transferred to 1 ml saline (0.9% NaCl). The biofilms were then homogenized to single cells using water sonication in ice bath for 15mins and 15-sec vortexing. Bacterial cells were serially diluted, plated on LB agar and incubated at 37 °C for 16 hrs. CFU ml<sup>-1</sup> was calculated as average number of colonies by the dilution factor and dividing by the volume. Experiments were performed with three replicates, and the results are shown as the mean $\pm$ s.d.

#### *Extraction of polysaccharides*

As previously described (3), Pel and Psl were extracted by growing  $\Delta pslBCD/p_{lac}-YedQ$ , and  $\Delta pelA/p_{lac}-YedQ$  static biofilms in 15 ml ABTGC in standard Petri dishes at 37 °C for 16 hrs. The biofilms were collected and separated from the supernatant by centrifugation at 10000 g for 5 mins. The cell pellet was re-suspended in 0.9% NaCl and homogenised with mild water sonication on ice (Elmasonic P120H, Power=50%, Frequency=37 KHz) for 5 mins to dislodge the surface-associated matrix from bacterial cells. The cells were then separated from the matrix by centrifugation at 13000 g for 3 mins, 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 hr and inactivation at 80 °C for 30 mins. The extract was then filtered with centrifugal filter (<3 kDa) at 15000 g for 5 mins to remove the bacterial metabolites. The extract was then lyophilized and re-suspended in 1 ml sterile ddH<sub>2</sub>O.

#### *C. elegans choice assay of purified exopolysaccharides and autoinducers*

Varying concentrations (0, 0.5, 1, 5 and 10  $\mu g\ ml^{-1}$ ) of Pel or Psl were mixed with 10  $\mu M$  OdDHL or BHL, and the solutions were added to the 0.2- $\mu m$  filter at one end of NGM plate, while its control was placed on the opposite end of the petri dish, followed by 16 hrs incubation at 37 °C. This allowed the OdDHL or BHL to diffuse through the filter, while Pel or Psl were retained on the filter. The filters (containing biofilms or chemicals) on opposite ends of petri dish were carefully peeled off from the NGM agar, leaving behind the diffused autoinducers in the agar. At least 50 individuals of adult *C. elegans* from 3 independent trials were transferred from the feeding plate to the center of the petri dish with wire picker. The animals were allowed to detect the secreted biofilm metabolites and migrate to the spot previously occupied by either control strain or test strain, where live animals localized at each spot were enumerated by 6 hrs.

A modified choice assay involving cellulase treatment to degrade the exopolysaccharides was also used, where Pel or Psl were pre-treated with 5  $\mu g\ ml^{-1}$  of cellulase for 24 hrs at 37 °C. The cellulase was heat-inactivated for 1 hr at 65 °C, where remaining Pel and Psl were purified as described in previous section, before use for choice assay.

*Exogenous addition of exopolysaccharides and autoinducers to non-producing strains*

The *P. aeruginosa*  $\Delta pelA\Delta pslBCD$  or *E. coli* OP50 cells from growth culture (described in the previous section on growth conditions) were washed with 0.9% NaCl and centrifuged at 13000 g for 3 mins. Varying concentrations (0, 0.5, 1, 5 and 10  $\mu\text{g ml}^{-1}$ ) of Pel or Psl were mixed with 10  $\mu\text{M}$  OdDHL or BHL, and the solutions were re-suspended in the cell pellet. The various combinations were used for the modified choice assays.

*C. elegans exploration assay*

The exploratory behavior of wild-type (N2) nematodes and *odr-10* (CX32) mutant was compared, where animals were transferred to NGM plates containing OP50 bacterial lawn. The trails of nematodes were monitored every 12 hrs till 48 hrs and recorded by enumerating the grids (5mm  $\times$  5mm size) covered by the trails.

*Bacterial population survival after C. elegans feeding*

To observe if nematodes attracted to the biofilm metabolite spot could reproduce and expand their population after the choice assay, the worms localized at their respective spots were transferred to fresh NGM plates containing OP50 lawn. The fresh NGM plates were further incubated at room temperature for 48 hrs to allow *C. elegans* feeding of bacterial prey. Bacteria on the lawns after 48 hrs of *C. elegans* L1 progeny growth were harvested for CFU assay, as described in earlier section.

*Statistical analysis*

The results were expressed as means  $\pm$  standard deviation. Data groups were compared using the one-way ANOVA and Student's t-test to evaluate associations between independent variables, and the P values were obtained. Three independent trials were conducted in triplicates for each experiment.

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**Author Contributions:**

S.L.C designed methods and experiments. S.C.Y, S.Y.L and Z.S carried out laboratory experiments, analyzed the data and interpreted the results. S.L.C, S.Y.L and S.C.Y wrote the paper. All authors have contributed to, seen and approved the manuscript.

**Competing interests:**

The authors declare no competing financial interests.

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## Figures:

**Figure 1: *C. elegans* could not detect secreted metabolites from exopolysaccharide-producing biofilms.** (A) Experimental design for modified choice assay. (B) *C. elegans* preferred biofilm metabolites of  $\Delta pelA$ ,  $\Delta pslBCD$  and  $\Delta pelA\Delta pslBCD$  mutants over PAO1 control,  $CI > 0.00$ . (C) *C. elegans* preferred biofilm metabolites of PAO1 control over  $\Delta wspF$  and PAO1/ $p_{lac}$ -*YedQ* mutants,  $CI < 0.00$ . (D) *C. elegans* preferred biofilm metabolites of  $\Delta wspF\Delta pelA$ ,  $\Delta wspF\Delta pslBCD$  and  $\Delta wspF\Delta pelA\Delta pslBCD$  mutants over  $\Delta wspF$  control,  $CI > 0.00$ . (E) *C. elegans* preferred biofilm metabolites of  $\Delta pelA\Delta pslBCD$  control over  $\Delta pelA\Delta pslBCD$  possessing exogenous Pel and Psl,  $CI < 0.00$ . Means and s.d. from triplicate experiments are shown.  $***p < 0.001$ , One-Way ANOVA.

**Figure 2: *C. elegans* detects bacterial autoinducers.** (A) *C. elegans* preferred pure autoinducers over saline control,  $CI > 0.00$ . (B) Pure OdDHL and BHL concentrations in the agar across distances. (C) BHL concentration in biofilms. (D) Biofilm-secreted BHL concentrations in agar across distances. (E) Increasing concentrations of Pel or Psl can trap 10  $\mu M$  BHL and prevent predator detection. Means and s.d. from triplicate experiments are shown. (F) Cellulase degradation of exopolysaccharides results in *C. elegans* detection of BHL, where *C. elegans* preferred cellulase-degraded pure exopolysaccharide with released 10  $\mu M$  BHL over pure exopolysaccharide with trapped 10  $\mu M$  BHL control,  $CI > 0.00$ .  $*p < 0.05$ ,  $***p < 0.001$ , n.s not significant, One-Way ANOVA.

**Figure 3: Exopolysaccharide is a public good for trapping autoinducers by non-exopolysaccharide-producers.** (A) *C. elegans* preferentially selected PAO1 control strain as compared to  $\Delta lasI\Delta rhII$  mutant,  $CI < 0.00$ . (B) Exogenous addition of Pel or Psl protected  $\Delta lasI\Delta rhII$  mutant from predator detection in the presence of BHL. (C) Exogenous addition of Pel or Psl protected *E. coli* OP50 from predator detection. (D) Exogenous addition of Pel or Psl protected *E. coli* OP50 from predator detection even in the presence of BHL. Means and s.d. from triplicate experiments are shown.  $***p < 0.001$ , One-Way ANOVA.

**Figure 4: *C. elegans* requires *ODR-10* to sense bacterial autoinducers.** (A) *C. elegans odr-10* mutant could not detect pure autoinducers. (B) *C. elegans odr-10* mutant could not differentiate mutant biofilm mutants from wild-type PAO1. (C) Experimental design for studying population growth of *C. elegans* after choice assay. (D) Higher population growth of wild-type N2 *C. elegans* which localized on metabolite spot left by  $\Delta pelA$ ,  $\Delta pslBCD$  and  $\Delta pelA\Delta pslBCD$  mutants relative to *odr-10* mutant. Means and s.d. from triplicate experiments are shown. \*\*  $p < 0.01$ , \*\*\* $p < 0.001$ , n.s not significant, One-Way ANOVA.

**Figure 5: Model of biofilms that employ biofilm matrix to hide QS autoinducers from predator (*C. elegans*) detection.**