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Feeding behaviour of *Caenorhabditis elegans* is an indicator of *Pseudomonas aeruginosa* PAO1 virulence.

Caenorhabditis elegans is commonly used as an infection model for pathogenesis studies in *Pseudomonas aeruginosa*. While the standard virulence assays rely on the slow and fast killing or paralysis of nematodes, here we developed a behaviour assay to monitor the preferred bacterial food sources of *C. elegans*. The type III secretion system is a well-conserved virulence trait that is not required for slow or fast killing of *C. elegans*. However, ΔexsE mutants that are competent for hypersecretion of ExoS, ExoT and ExoY effectors were avoided as food sources in binary assays. Conversely, mutants lacking the secretion machinery or type III effectors were preferred food sources for PAO1. In binary feeding assays, both food sources were ingested and observed in the gastrointestinal tract, but non-preferred food sources were ultimately avoided. Next we developed a high throughput feeding behaviour assay to test a library of 2370 transposon mutants in order to identify preferred food sources. After primary and secondary screens, 37 mutants were identified as preferred food sources, which included mutations in many known virulence genes and that showed reduced virulence in the slow killing assay. We propose that *C. elegans* feeding behaviour can be used as a sensitive indicator of virulence for bacterial strains that have moderate worm killing activity.

Feeding behaviour of *Caenorhabditis elegans* is an indicator of *Pseudomonas aeruginosa* PAO1 virulence.

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Abstract

Caenorhabditis elegans is commonly used as an infection model for pathogenesis studies in *Pseudomonas aeruginosa*. While the standard virulence assays rely on the slow and fast killing or paralysis of nematodes, here we developed a behaviour assay to monitor the preferred bacterial food sources of *C. elegans*. The type III secretion system is a well-conserved virulence trait that is not required for slow or fast killing of *C. elegans*. However, Δ *exsE* mutants that are competent for hypersecretion of ExoS, ExoT and ExoY effectors were avoided as food sources in binary assays. Conversely, mutants lacking the secretion machinery or type III effectors were preferred food sources for PAO1. In binary feeding assays, both food sources were ingested and observed in the gastrointestinal tract, but non-preferred food sources were ultimately avoided. Next we developed a high throughput feeding behaviour assay to test a library of 2370 transposon mutants in order to identify preferred food sources. After primary and secondary screens, 37 mutants were identified as preferred food sources, which included mutations in many known virulence genes and that showed reduced virulence in the slow killing assay. We propose that *C. elegans* feeding behaviour can be used as a sensitive indicator of virulence for bacterial strains that have moderate worm killing activity.

Keywords

Caenorhabditis elegans, *Pseudomonas aeruginosa*, type III secretion, nematode feeding behaviour, high throughput virulence model, food preferences

Introduction

C. elegans is an important model organism for developmental biology and infectious diseases research. The nematode has been used for numerous studies as an infection host for *Pseudomonas aeruginosa* and many other bacteria (Sifri, Begun & Ausubel, 2005). *C. elegans* is a bacteriovore that forages for bacteria in rotting plants and soil. When *C. elegans* is fed a lawn of *P. aeruginosa* PA14, the gut is colonized and numerous virulence factors contribute to worm death over a period of days, also known as slow killing (Tan, Mahajan-Miklos & Ausubel, 1999; Feinbaum et al., 2012). Defects in virulence of transposon mutants fed to synchronized worms are determined by decreased nematode killing kinetics relative to the wild type strain. After ingestion, *P. aeruginosa* caused gut distension, the production of biofilm-like extracellular matrix in the lumen, penetrated the intestinal barrier and invaded epithelial cells to some extent (Irazoqui et al., 2010). *P. aeruginosa* PA14 uses a wide range of virulence phenotypes in the slow killing assay (Feinbaum et al., 2012), but when PA14 is grown on rich, high osmolarity medium, the fast killing pathway does not require live bacteria and is due to the production of toxic phenazine compounds (Cezairliyan et al., 2013). *P. aeruginosa* strains display a range of virulence phenotypes and PAO1 is among the strains with moderate slow killing activity (Lee et al., 2006). However, PAO1 was shown to induce a rapid, paralytic killing mechanism dependent on hydrogen cyanide production (Gallagher & Manoel, 2001).

Several high throughput methods have been developed to screen large transposon mutant libraries to identify virulence genes. Garvis *et al.* developed a high throughput screen of 2200 transposon mutants for defects in *C. elegans* killing using a liquid assay and 24 hours exposure to *P. aeruginosa* (Garvis et al., 2009). Kirienko *et al.* also used liquid killing assays to show that *P. aeruginosa* does not require phenazines, quorum sensing or colonization, but does require the

siderophore pyoverdinin to kill *C. elegans* in liquid conditions (Kirienko et al., 2013). Pyoverdinin production was also required for the red death killing of *C. elegans* when *P. aeruginosa* PAO1 is grown under phosphate limiting conditions, along with the PhoB two-component response regulator and the MvfR-PQS quorum sensing regulator (Zaborin et al., 2009).

While killing assays measure the effect of single food sources on worm lethality, it is known that *C. elegans* does have a feeding preference for certain species of bacterial isolates (Zhang, Lu & Bargmann, 2005; Abada et al., 2009; Frey et al., 2010). The choice index assays allow one to compare the relative preference of *C. elegans* for OP50 to other bacterial food sources in binary assays (Zhang, Lu & Bargmann, 2005; Abada et al., 2009). Bacterial isolates that are preferred over OP50 as food sources were shown to increase the life span and reproductive fitness of *C. elegans* (Abada et al., 2009; Frey et al., 2010). Binary assays have also shown that *C. elegans* learns to avoid pathogenic bacteria, if the worms are exposed to the pathogen prior to a binary choice feeding behaviour experiment (Zhang, Lu & Bargmann, 2005). Worms may be attracted to certain diffusible odorants and repelled by others (Bargmann, Hartwig & Horvitz, 1993; Schulenburg & Ewbank, 2007), however, *Bacillus nematocida* both attracts and kills *C. elegans*, thereby acting as a nematode predator (Niu et al., 2010). *C. elegans* feeding behaviour, sensing and decision making regarding food sources is complex and involves multiple neural pathways (Schulenburg & Ewbank, 2007; Sengupta, 2013).

While the type III secretion system is considered a crucial virulence factor for *P. aeruginosa* (Hauser, 2009), it does not seem to be required for PA14 killing of *C. elegans* (Wareham, Papakonstantinou & Curtis, 2005). In this report, we revisited the role of the type III secretion system using a panel of strains that were competent for hypersecretion or

defective in all three PAO1 effectors ExoS, ExoT and ExoY. We show that hypersecreting strains are avoided and type III secretion deficient strains are preferred. Based on this subtle worm feeding behaviour, we developed a high throughput feeding assay that led to the identification of mutants that served as preferred food sources, many of which were known *P. aeruginosa* virulence factors. These observations suggest that *C. elegans* food preferences are a subtle indicator of virulence defects in *Pseudomonas aeruginosa* PAO1.

Materials and Methods.

Strains and growth conditions. The *Caenorhabditis elegans* N2 Bristol strain was used for bacterial infections and feeding assays. The *tph-1* mutant worm is deficient in tryptophan hydroxylase required for the biosynthesis of serotonin (provided by Dr Jim McGhee). *Escherichia coli* OP50 was used as a non-pathogenic food source for cultivating *C. elegans*. OP50 was grown overnight in LB medium at 37°C and 10 µl (1x10⁷cfu/ml) were spread as bacterial lawn and nematode food source on NGM plates. The NGM medium is composed of double distilled water, 0.25% (w/v) Bacto-Peptone (BD), 0.3% (w/v) NaCl, and 2% (w/v) Bacto-Agar (BD), 5 µg/ml cholesterol, 1 mM MgSO₄, 25 mM KH₂PO₄ (pH 6) and 1 mM CaCl₂. Wild type *P. aeruginosa* PAO1 and all mini-Tn5-lux transposon mutants were previously described (Lewenza et al., 2005). The panel of type III secretion mutant strains and the wild type PAO1F are described in Table 1 (provided by Dr Arne Rietsch).

***C. elegans* feeding behaviour assays.** Two adult *C. elegans* worms were transferred to NGM plates with a lawn of *E. coli* OP50, and incubated 3 days at 25°C until the new larvae reached the L4 phase. Ten µl (1x10⁷cfu/ml) of bacterial food sources were spotted on SK plates and grown overnight. Twenty L4 worms were then transferred to 6 cm SK plates containing the pre-grown

93 *P. aeruginosa* food sources. The SK medium is similar to NGM but contains 0.35% (w/v) Bacto-
94 Peptone (BD). SK plates with two, three or four *P. aeruginosa* food sources were monitored
95 throughout a 3-5 day period to monitor the preferred food source by counting the number of
96 worms in each bacterial colony. Feeding assays were performed at 25°C. The paired t-test was
97 used to compare the number of worms that selected mutant or wild type bacterial food sources at
98 each time point. Images of colonies and worms were captured with a Motic DM143 digital
99 microscope.

101 ***Fluorescence microscopy of C. elegans.*** L4 worms were given the choice of Rfp-tagged PAO1
102 expressing pCHAP6619 (Lewenza, Mhlanga & Pugsley, 2008) or *exoS::GL3 ΔexsE* strain as an
103 *exoS::gfp*-tagged food source. Worms were transferred from Rfp or Gfp-tagged colonies to
104 mounting media on microscope slides and visualized using a Leica DMI4000 B inverted
105 microscope equipped with an ORCA R2 digital camera. The following excitation and emission
106 filters were used to monitor red and green fluorescence, respectively (Ex 555/25; Em 605/52; Ex
107 490/20; Em 525/36). The Quorum Angstrom Optigrid (MetaMorph) acquisition software was
108 used for image acquisition with a 63 × 1.4 objective and deconvolution was performed with
109 Huygens Essential (Scientific Volume Imaging B.V.).

111 ***High throughput assay to identify preferred bacterial food sources.*** We previously constructed
112 a mini-Tn5-lux transposon mutant library and mapped the transposon insertion site in 2370
113 individual mutants (Lewenza et al., 2005). This collection of transposon mutants was arrayed and
114 grown overnight in LB medium in 25 X 96-well microplates. A 48-pin stamp was used to stamp
115 transfer ~5 µl of liquid culture onto a 6 X 8 grid of colonies on 15 cm SK agar plates and grown
116 overnight. Ten L4 worms were added to each side of the SK plates (20 worms total) and were

allowed to eat and reproduce over the course of 3-5 days at 25°C. Each plate with 48 food sources was monitored daily for the disappearance of specific colonies, which we identified as preferred food sources. After eating of preferred colonies, the nematodes would reproduce to high numbers and eat all the bacterial colonies to completion. Mutants identified in the primary screen of 2370 strains were retested in secondary screens to confirm the preferred food source phenotype. In one method, 6 mutants were arrayed in 6 specific positions within a 6 X 8 grid and surrounded by wild type PAO1. Alternatively, individual mutants were positioned in 3 consistent well positions in the middle of a 6 X 8 grid of wild type PAO1. Ten L4 worms were added to the both sides of the plates (20 worms total), which were monitored over the course of 3 to 5 days to determine the food preference.

Growth defect analysis. All mutants that served as preferred food sources for *C. elegans* were tested for growth defects in LB and SK liquid media. Briefly, each strain was grown overnight in 100 µl LB cultures in 96-well microplates and diluted 1/500 into 100 µl of fresh LB or SK liquid media and OD₆₀₀ values were monitored every 20 min throughout 18 hours growth at 37°C, without shaking. Mutations that caused growth defects in SK growth medium were excluded from further analysis.

Worm chemotaxis assays. Bacterial cultures were grown overnight and supernatants were collected. Ten µl of culture supernatant were spotted on 6 cm SK plates at 3 cm distances from the origin, where 15 L4 worms were transferred to SK plates. The plates were monitored throughout 2 hours to count the number of worms that moved into the dried spot of bacterial supernatant. Each supernatant was tested two times.

Slow killing assays. As previously described (Powell & Ausubel, 2008), ten μl (1×10^7 cfu/ml) of bacterial cultures were transferred to 6 cm SK plates and spread to form a bacterial lawn. Thirty L4 stage worms were transferred from NGM to SK plates containing the pre-grown bacterial lawn and incubated at 25°C. SK plates were prepared with 25 $\mu\text{g/ml}$ of 5-fluoro-2'-deoxyuridine (FUDR), a eukaryote DNA synthesis inhibitor that prevents the growth of egg offspring during the experiment. Worms were monitored under a dissection microscope over a period of 10 days to detect unresponsive worms.

Results and Discussion.

Ingestion and food preference in binary feeding assays with *C. elegans*. Regulation of the type III secretion system involves a negative regulator called ExsE (Rietsch et al., 2005). In ΔexsE mutants, there is an increased expression of *exoS* regardless of the presence of inducing conditions (Rietsch et al., 2005). The ΔexsE mutant demonstrated increased cytotoxicity to host cells, presumably due to the increased gene expression of type III secreted effectors, which were injected into host cells upon contact (Rietsch et al., 2005). Similarly, there was increased secretion of all three effectors ExoT, ExoY and ExoS into the culture supernatant during growth in inducing, low calcium conditions (Rietsch et al., 2005). We were interested to determine if the ΔexsE mutant that is competent for hypersecretion of the type III effectors had an effect on *C. elegans* feeding behaviour. Using a binary feeding assay, L4 stage nematodes preferred wild type PAO1 over the ΔexsE strain, which suggested that hypersecretion of type III effectors was detected and avoided by the worm.

In addition to counting the number of worms that chose a given food source, *C. elegans* was fed an Rfp-tagged wild type PAO1 or Gfp-tagged, ΔexsE *exoS-gfp* strain in binary assays. At

early time points, worms that were removed from wild type PAO1 colonies were shown to have exclusively RFP-tagged bacteria in the gastrointestinal tract (Fig 1B). Worms taken at early time points from the *ΔexsE* *exoS-gfp* strain had exclusively GFP-tagged bacteria in the GI tract (Fig 1C). The worm body was autofluorescent in both green and red channels, but the GI tract was obviously distended with fluorescent red or green bacteria (Fig 1). This indicates that worms ingested both food sources and did not rely exclusively on olfactory cues and aversion. At later time points, this was confirmed as worms were shown to have both Rfp and Gfp-tagged bacteria in the GI tract (Fig 1D). We concluded that the worm ingested both possible food sources, and ultimately preferred the wild type and avoided the *ΔexsE* mutant. Interestingly, not all worms chose one of the two food sources (Fig 1A), possibly due to the virulence phenotypes of either strain. As exposure time increased, the number of worms that chose neither food source decreased (Fig 1A), although worms were observed that did not have any bacteria in the GI tract (1E).

Type III secretion mutants are preferred food sources. We reasoned that if type III hypersecretion strains were avoided, than secretion defective strains might be preferred. Indeed, *C. elegans* preferred strains that were defective in the type III secretion machinery (*ΔexsE* *ΔpscD*) and that were defective for all three type III secretion effectors (*Δ3TOX*) (Fig 2A). After longer periods of feeding, between 3 and 5 days, the *ΔpscD* food source was the most preferred as it was the colony first consumed (Fig 2C). In an attempt to compare the potential toxicity of type III effectors, we showed that worms exposed to strains that hypersecrete ExoS, ExoY or ExoT, consistently chose the ExoY secreting strain as a preferred food source (Fig 2B). ExoY is an adenylate cyclase and our findings are comparable with a report showing that ExoY was not

required for in vitro cytotoxicity and had no impact on dissemination during infection (Lee et al., 2005).

High throughput feeding behaviour assays.

Given the results observed with strains defective

for type III secretion, we wanted to screen a large collection of mutants to find additional

preferred food sources as an approach to identify candidate virulence genes. We previously

constructed a large library of mini-Tn5-*lux* mutants with mapped insertion sites, which were

arrayed into a library of 2370 mutants in 96-well microplates (Lewenza et al., 2005). Using

standard petri dishes with SK agar, we stamped liquid LB cultures onto a grid of 48 colonies (6 X

8), and introduced 20 L4 stage nematodes. The plates were incubated at 25°C and observed daily

to identify colonies that were preferentially eaten to completion. In the primary screen of 2370

mutants, we identified 191 strains that were preferred food sources. We developed secondary

screens to confirm this phenotype. In one method, we arranged 6 unique preferred food sources

within a grid of 48 colonies, with PAO1 in all other positions (Fig 3A). This method did confirm

the preferred feeding source phenotype of some mutants, but not all. Since the worm was given a

choice of multiple preferred food sources, there may have been competition for the most

preferred. To reduce the competition, we used another secondary screen where individual

candidates were situated in triplicate positions within a 48-grid of wild type PAO1 (Fig 3B).

Using these two secondary screens, we confirmed the preferred food source phenotype of 37

mutants (Table 2). After sufficient incubation time, the nematodes would reproduce to high

numbers and eat all the bacterial colonies to completion.

Preferred food sources are not due to increased attraction of *C. elegans*.

A simple explanation for preferred feeding is that the bacterial mutants had an altered production of

odorants. For example, they may not produce a repellent or have increased production of an attractant. To test this hypothesis, we compared the worm migration towards the supernatants from wild type PAO1 or preferred food sources in a binary chemoattraction assay. Culture supernatants were collected and spotted on SK plates at equal distances from where 20 L4 worms were transferred. Approximately one third of the 18 supernatants tested from preferred sources were attractive to *C. elegans*, one third of supernatants were repellent, and one third were no different from the wild type strain. Representative examples of these three patterns of behaviour in chemoattraction assays are shown in Fig 4. We concluded that preferred feeding was not due exclusively to olfactory cues and altered odorant production.

***tph-1* worms demonstrated preferred feeding behaviour.** The *tph-1* strain is defective for the enzyme tryptophan hydroxylase, which is the rate-limiting step in the biosynthesis of serotonin. This neurotransmitter was previously shown to be important for aversive learning and avoidance of *P. aeruginosa* (Zhang, Lu & Bargmann, 2005). Naive worms have a preference for *P. aeruginosa* over *E. coli* OP50, but learn to avoid virulent *P. aeruginosa* strains in binary choice assays if they were pre-exposed to *P. aeruginosa* for 4 hours prior to the food choice experiment (Zhang, Lu & Bargmann, 2005). The avoidance behaviour is specific to the strains that were exposed to the worms, and requires the serotonin neurotransmitter pathway (Zhang, Lu & Bargmann, 2005). Here we tested *tph-1* worms for their preference for either the type III hypersecretion Δ *exsE* strain of *P. aeruginosa* or *E. coli* OP50. At early time points (5 hours), wild type N2 worms preferred *P. aeruginosa* Δ *exsE*, but preferred OP50 after 24-48 hours exposure (Fig 5A). The *tph-1* mutant strain did not have any preference at 5 hours, but also demonstrated a preference for OP50 between 24 and 48 hours (Fig 5A).

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In our feeding behaviour assay with 48 colonies, the preferred food sources are detected after 3 to 5 days of exposure (Fig 3). Given the rapid, aversive learning behaviour after 4 hours of pathogen pre-exposure (Zhang, Lu & Bargmann, 2005), we wanted to determine if the *tph-1* worms were also capable of detecting the food sources preferred by N2. We tested a subset of the N2 preferred food sources and showed that *tph-1* worms were still capable of detecting and eating those preferred sources to completion before moving on to eat the wild type PAO1 (Fig 5B). Despite the increased exposure time in the feeding behaviour assay with 48 colonies, and the possibility for aversive learning to occur by sampling wild type PAO1 during that time, the serotonin neurotransmitter is not required to detect preferred food sources.

Preferred food sources for *C. elegans* included mutants in many known virulence factors.

Table 2 summarizes the transposon insertion sites of genes that led to the preferred food source status for *C. elegans*. The genes identified in this screen can be grouped into the following categories: known virulence factors, virulence regulatory systems, nutrient utilization and metabolism, and hypothetical proteins. All of the genes identified in PAO1 as having a preferred food source phenotype are present in PA14 but were not identified in the genome-wide screen for slow killing determinants in PA14. However, transposon mutants to approximately 75% of the genes shown to be required for slow killing by PA14 were not present in our PAO1 mutant library (eg. *rhlR*, *lasIR*, *vfr*, *vqsR*, *gacAS*, *pchHI*, *gshAB*, *prpBC*) (Feinbaum et al., 2012). Mutations in the *pqs* biosynthesis and type IV pili genes result in slow killing defects (Feinbaum et al., 2012) and led to preferred food source status (Table 2).

Mutations in genes encoding the PQS biosynthesis genes, type IV pili and the TypA GTPase had a preferred food source phenotype, and all were previously recognized as virulence

factors in *P. aeruginosa* (Neidig et al., 2013). Among the known regulators of virulence, we identified the PprB, PhoQ, PqsR and CifR regulatory systems (Cao et al., 2001; MacEachran, Stanton & O'Toole, 2008; Gooderham et al., 2009; de Bentzmann et al., 2012). The *C. elegans* virulence screens frequently identify global regulators and two-component system regulators (Sifri, Begun & Ausubel, 2005), probably due to the pleiotropic effects of these mutations, given the large number of virulence genes controlled by these systems. Both PqsR and PprB are known regulators of the *pqsABCDE* biosynthesis genes (Cao et al., 2001; de Bentzmann et al., 2012), which were previously shown to be involved in the *C. elegans* red death and slow killing phenotypes (Zaborin et al., 2009; Feinbaum et al., 2012). PhoQ is a two-component sensor that responds to limiting Mg^{2+} and controls numerous genes including antimicrobial peptide resistance modification to LPS (Macfarlane et al., 1999), and is required for virulence in plant and chronic rat lung infections (Gooderham et al., 2009). The CifR repressor controls the Cif secreted toxin that reduces the apical expression of CFTR and chloride secretion in epithelial cells (MacEachran et al., 2007; MacEachran, Stanton & O'Toole, 2008). PA14, but not PAO1, expresses Cif activity (Swiatecka-Urban et al., 2006), suggesting that the CifR mutant phenotype in this assay is independent of Cif activity. The lack of significant overlap in genes required for both the slow killing and preferred food source phenotypes suggests that these assays are distinct measures of virulence effects on *C. elegans*.

Preferred food sources for *C. elegans* included mutants in nutrient acquisition pathways.

Several additional transcriptional regulators were identified in this screen including *PA0056*, *PA0929-PA0930*, *PA4983* and *dctD* (Table 2). Intergenic insertions between PA0120-PA0121 likely disrupted the downstream PA0121, an uncharacterized transcriptional regulator. It is unclear what gene is affected by an intergenic insertion between PA4353-PA4354, due to their

divergent orientation. The functions of *PA0056*, *PA4983* and *PA0929-PA0930* are currently unknown, and the latter two-component system is adjacent to *gacS*. The AgtS two-component sensor regulates an ABC transporter that is required for the uptake of δ -aminovalerate (AMV) and γ -aminobutyrate (GABA) (Chou, Li & Lu, 2014). The cognate response regulator AgtR is also required to sense peptidoglycan shed from Gram-positive bacteria, leading to increase virulence factor production, killing of *S. aureus* and enhanced fruit fly killing during mixed infection (Korgaonkar et al., 2013).

Additional genes were found that are required for the uptake and utilization C-4 dicarboxylates, such as the TCA intermediates malate, fumarate and succinate as carbon sources. The DctD two-component response regulator controls expression of the ABC transporters that take up C-4 dicarboxylates (Valentini, Storelli & Lapouge, 2011), and a mutation in this gene led to a preferred food source for *C. elegans* (Table 2). In addition, there were mutations in multiple ABC transporters that led to preferred food source status, including transporters involved in the uptake of dipeptides, quaternary ammonium compounds (QAC), zinc, as well as genes involved in amino acid metabolism (Table 2). Mutations in genes required for carbon compound catabolism (*aceF*, *mmsB*) or energy generation (*nqrB*,) also led to preferred food source status. These observations suggest that *P. aeruginosa* requires the ability to utilize dicarboxylates, amino acids and QACs as nutrient sources in the nematode gut for full virulence. Preferred bacterial food sources are associated with increased *C. elegans* life span and reproductive fitness (Abada et al., 2009; Frey et al., 2010). To determine if preferred food sources were less virulent than PAO1, we tested a panel of 20 mutants identified as preferred food sources for defects in slow killing of *C. elegans*. Interestingly, 14/20 strains had slight decreases in slow killing kinetics relative to the wild type PAO1, but were more virulent than the *E. coli* OP50 food source (Fig 6).

Conclusions

We describe a method to detect preferred food sources of *C. elegans* and propose this as a new strategy to identify *P. aeruginosa* virulence factors. Preferred food sources were defective in several known virulence factors of *P. aeruginosa*, some of which were specifically shown to contribute to *C. elegans* slow killing. This approach was also useful in demonstrating a preference for strains that were defective for the type III secretion system, suggesting a subtle role in virulence that cannot be detected in the slow killing assay (Wareham, Papakonstantinou & Curtis, 2005). Recently, a similar high throughput feeding behaviour assay was used to screen transposon mutants of *P. fluorescens* NZI7 in order to identify the repellants that deter grazing by *C. elegans* (Burlinson et al., 2013). These two studies highlight the potential of the nematode's ability to make decisions and determine less virulent or edible food sources. The serotonin neurotransmitter is not required by *C. elegans* to detect preferred *P. aeruginosa* food sources and it will be important to understand the mechanisms of differentiating bacterial food sources.

It is interesting to note that comparisons between this study and other reports of *P. aeruginosa* virulence factors in *C. elegans* reveal little overlap in the bacterial requirements for the various mechanisms of worm killing (Tan, Mahajan-Miklos & Ausubel, 1999; Gallagher & Manoil, 2001; Garvis et al., 2009; Feinbaum et al., 2012; Kirienko et al., 2013; Zaborin et al., 2009). It will be important to determine if these differences are due to genetic or regulatory variation in bacterial or worm strains, different bacterial growth conditions, or if this reflects a challenge to using the *C. elegans* system. The observation that many *P. aeruginosa* virulence factors in *C. elegans* are conserved in the amoeba, fruit fly, plant, mice and chronic rat lung

models of infection justifies the use of this relatively simple infection host that is amenable to high throughput screening and is useful to identify new antibiotics that enhance nematode survival from bacterial infection (Moy et al., 2009).

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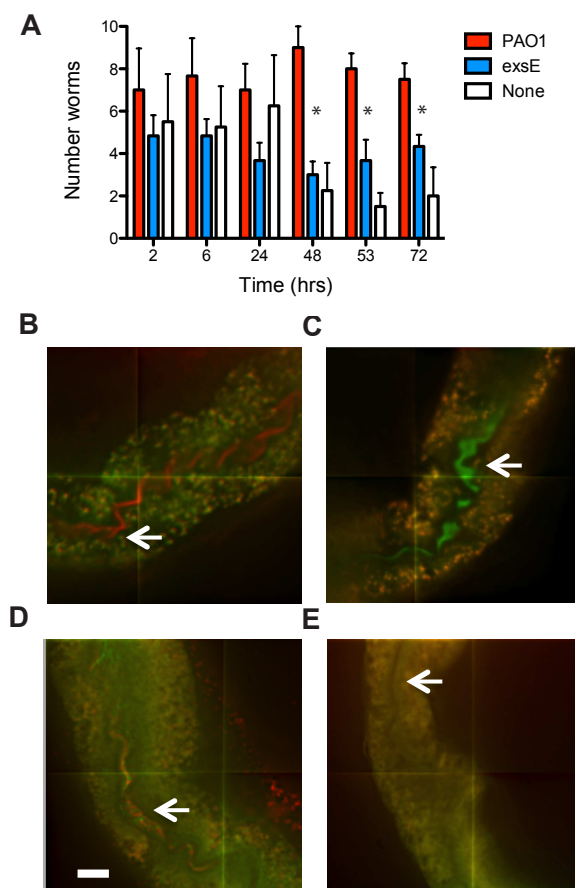


Figure 1. *C. elegans* ingests but ultimately avoids a *P. aeruginosa* $\Delta exsE$ mutant that is competent for hypersecretion of type III effectors. (A) In binary feeding assays, *C. elegans* was given a choice of two possible *P. aeruginosa* food sources on SK agar plates. The feeding preference was monitored by counting the worms in or near either food source. Values shown are the averages and SEM of six experiments where the number of total worms equals 120. Asterisks indicate a significant difference between the mutant and wild type ($p < 0.01$). Worms were removed from bacterial food source colonies at various times to detect either Rfp or Gfp-labelled bacteria in the GI tract. (B) At early time points (6 hours), worms selected from PAO1-Rfp colonies showed only red fluorescence in the gastrointestinal (GI) tract and (C) worms selected from $\Delta exsE$ *exoS-gfp* colonies showed only green fluorescence in the GI tract. (D) At later time points (48 hrs), worms were shown to have mixed Gfp and Rfp-labelled bacteria in the GI tract, indicating that worms had sampled and ingested both food sources. (E) Worms selected from outside the colonies avoided eating, as neither food source was observed in their GI tracts. White arrows point to the GI tract. Scale bar, 15 μ M.

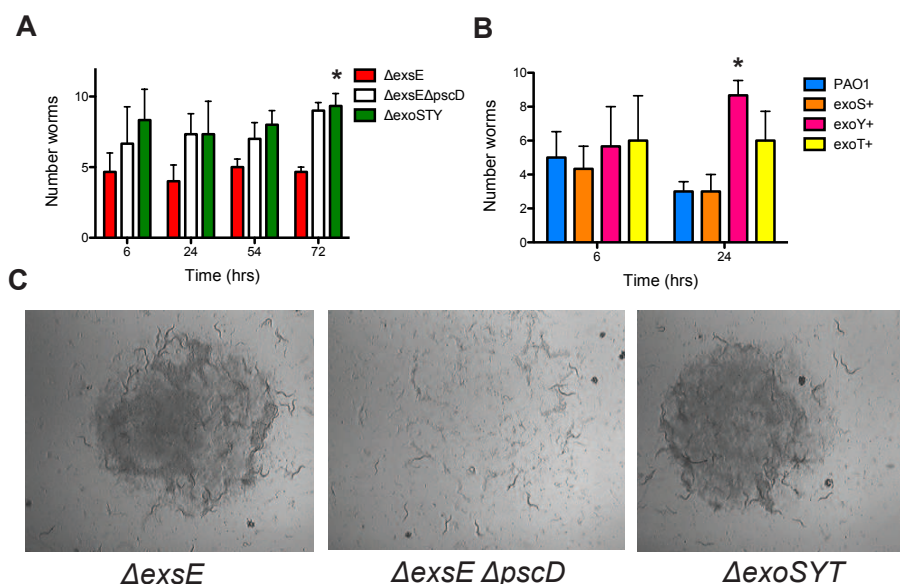


Figure 2. *C. elegans* prefers *P. aeruginosa* mutants defective for type III secretion secretion as food sources. (A). Worms were given the choice of three food sources: an $\Delta exsE$ mutant that is competent for type III hypersecretion, a secretion defective $\Delta exsE\Delta pscD$ double mutant and a triple effector mutant $\Delta exoSYT$. The feeding preference was monitored by counting the worms in or near the respective colonies. Values shown are the averages and SEM of three experiments where the number of total worms equals 60. **(B)** Feeding preference of worms given the choice of PAO1 wild type, and strains competent for hypersecretion of either ExoS, ExoY, or ExoT. Values shown are the averages and SEM of three experiments where the number of total worms equals 60. Asterisks indicate a significant difference between the preferred strain and wild type ($p < 0.01$). **(C)** After 5 days of feeding, the $\Delta exsE\Delta pscD$ mutant was the first colony eaten to completion.

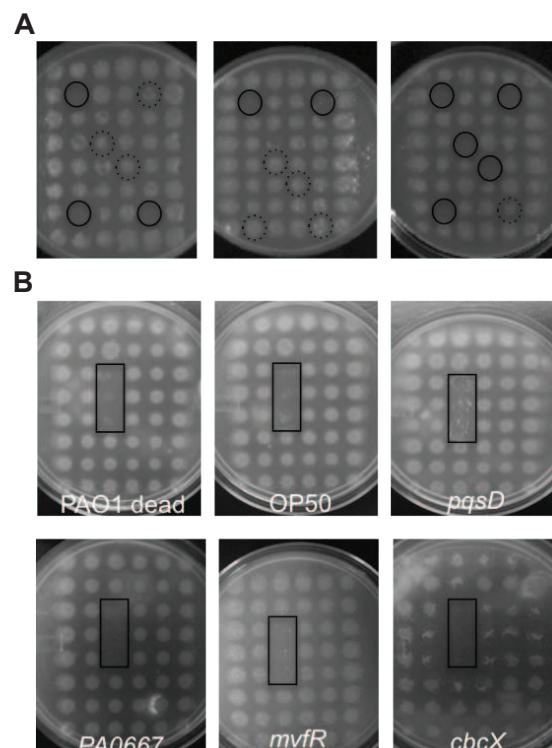


Figure 3. Preferred feeding behaviour in high throughput assays with a choice of 48 food sources for *C. elegans*. After the initial screen for mutants that acted as preferred food sources, secondary screens were performed to confirm the phenotype. **(A)** Six different preferred food sources were spotted at specific positions within a 48-grid of wild type PAO1 colonies. Solid circles indicate preferred food sources and dashed circles highlight partially eaten or uneaten colonies. **(B)** Individual food sources were grown in triplicate at consistent positions within a 48-grid of wild type PAO1 colonies. As positive controls, heat killed PAO1 or *E. coli* OP50 were spotted in triplicate. The black rectangles highlight the triplicate positions of preferred food sources, which include transposon insertion mutants in *pqsD*, *PA0667*, *mvfR* or *cbcX*. All images were captured between 3 to 5 days after the addition of 20 L4 nematodes.

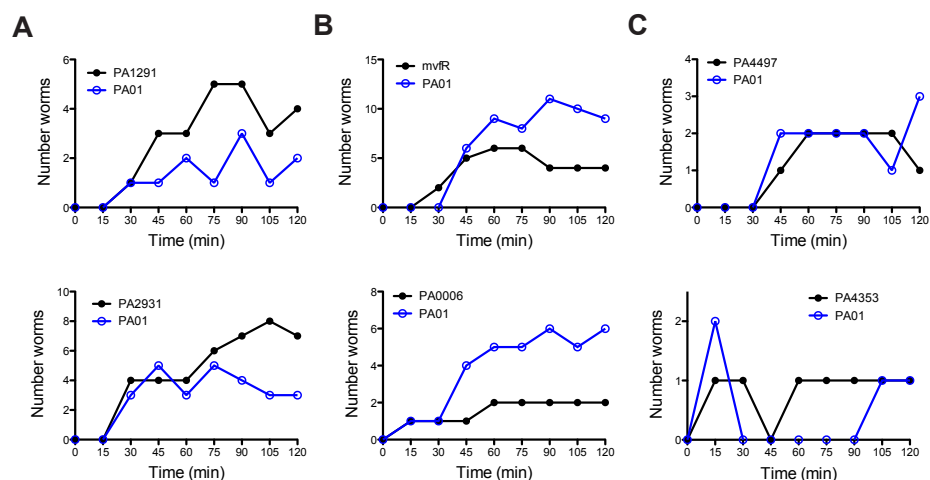


Figure 4. *C. elegans* is not chemoattracted universally towards bacterial culture supernatants from preferred food sources. Bacterial culture supernatants from a panel of preferred food sources were spotted onto SK plates and the migration behaviour of *C. elegans* was monitored throughout 2 hours. Nematodes were either (A) attracted to, (B) repelled or (C) were neutral upon exposure to the culture supernatants from preferred food sources.

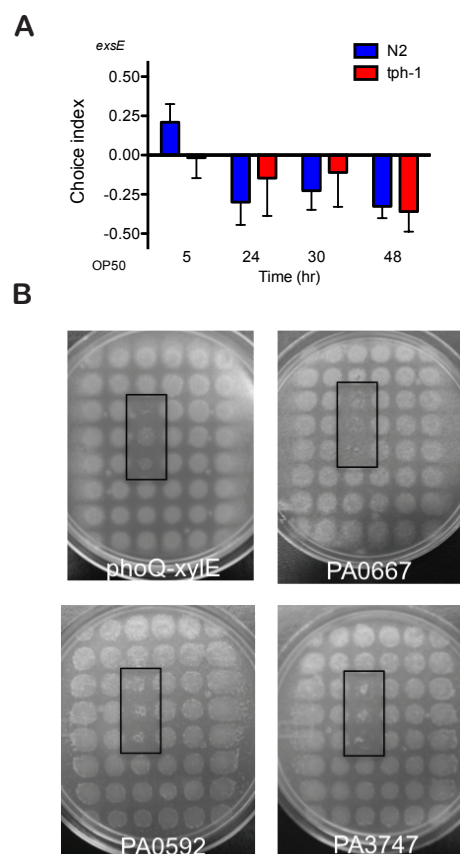


Figure 5. *C. elegans* *tph-1* strains are capable of avoidance and preferred feeding behaviours. (A) Wild type N2 and *tph-1* nematodes were given the choice of *E. coli* OP50 and the Δ *exsE* mutant and the choice index was determined throughout 48 hours. A positive choice index is an attraction to *P. aeruginosa* Δ *exsE* and a negative choice index is an attraction to OP50. Values shown are the averages and SEM of three experiments where the number of total worms equals 60. (B) The *tph-1* nematodes were tested in high throughput feeding assays where preferred food sources were embedded in a 48-grid of wild type PAO1 colonies. The black rectangles highlight the position of preferred food sources, which included mutants in *phoQ*, PA0667, PA0592 and PA3747. All images were captured between 3 to 5 days after the addition of 20 *tph-1* nematodes.

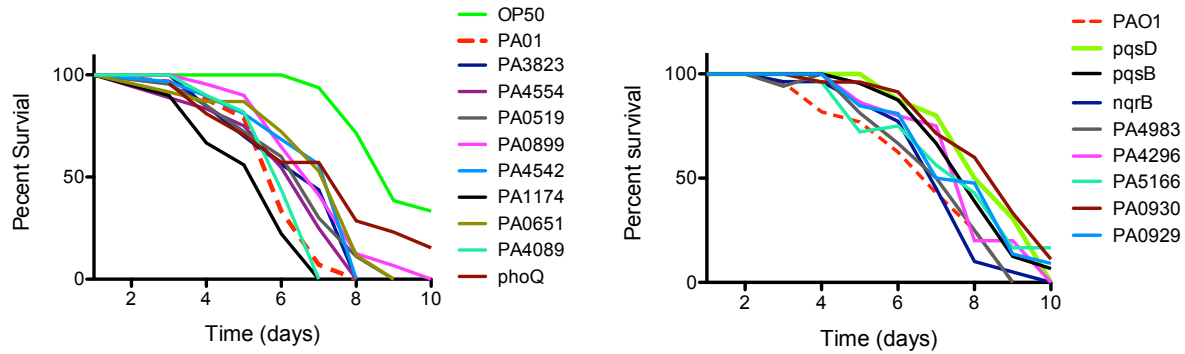


Figure 6. Preferred food sources have modest decreases in slow killing of *C. elegans*. A panel of 17 strains that served as preferred food sources were tested for virulence defects against *C. elegans* in the standard slow killing assay. Killing kinetics of each strain was measured over a period of 10 days and compared to wild type PAO1 infection and non-pathogenic *E. coli* OP50 as the sole food source. For each experiment, 25 L4 worms were fed a single food source and dead worms were scored over time.

Table 1. Type III secretion strains used in this study.

Strains	Description	Source
PAO1F	Wild type PAO1 from Alain Filloux's lab	Arne Rietsch
$\Delta exsE$	Loss of negative regulator of type III secretion	Arne Rietsch, (Cisz, Lee, & Rietsch, 2008)
$\Delta exsE \Delta pscD$	Mutation in type III secretion machinery	Arne Rietsch, unpublished
$\Delta exsE \Delta exoS \Delta exoT$		Arne Rietsch, (Cisz, Lee, & Rietsch, 2008)
$\Delta exoY$	Triple mutant " $\Delta 3TOX$ " for type III effectors	Arne Rietsch, (Cisz, Lee, & Rietsch, 2008)
$\Delta exsE exoT \Delta exoY (S+)$	Competent for hypersecretion of ExoS	Arne Rietsch, (Cisz, Lee, & Rietsch, 2008)
$\Delta exsE \Delta exoS \Delta exoY (T+)$	Competent for hypersecretion of ExoT	Arne Rietsch, (Cisz, Lee, & Rietsch, 2008)
$\Delta exsE \Delta exoS \Delta exoT (Y+)$	Competent for hypersecretion of ExoY	Arne Rietsch, (Cisz, Lee, & Rietsch, 2008)
$\Delta exsE exoS::GL3$	<i>exoS::gfp</i> reporter in $\Delta exsE$ background	Arne Rietsch, (Cisz, Lee, & Rietsch, 2008)

Table 2. PAO1 transposon mutants that are preferred food sources to *C. elegans*.

Mutant ID	Insertion site	Gene	PA	Gene Description	Screen ^a
11_B8	intergenic	<i>PA0120-21</i>		Transcriptional regulator-Transcriptional regulator	B
17_B11	intergenic	<i>PA4353-54</i>		Hypothetical - Transcriptional Regulator	B
20_D11	intergenic	<i>PA0006-07</i>		Histidinol phosphatase-Hypothetical	B
11_B4	gene	<i>PA0056</i>	PA0056	Probable transcriptional regulator	B
12_G5	gene	<i>PA0578</i>	PA0578	Conserved hypothetical protein	B
16_E10	gene	<i>ksgA</i>	PA0592	rRNA (adenine-N6,N6)-dimethyltransferase	B
23_C9	gene	<i>agtS</i>	PA0600	Two-component sensor	B
83_C1	gene	<i>trpC</i>	PA0651	Indole-3-glycerol-phosphate synthase	A
52_D11	gene	<i>PA0667</i>	PA0667	Putative metalloproteinase	B
37_C7	gene	<i>PA0929</i>	PA0929	Two-component response regulator	A
52_B2	gene	<i>PA0930</i>	PA0930	Two-component sensor	A
23_D6	gene	<i>pqsB</i>	PA0997	3-oxoacyl-[acyl-carrier-protein] synthase III	A
32_D10	gene	<i>pqsD</i>	PA0999	3-oxoacyl-[acyl-carrier-protein]	A, B
76_C11	gene	<i>pqsE</i>	PA1000	Quinolone signal response protein	A
44_H6	gene	<i>myfR</i>	PA1003	Transcriptional regulator of PQS synthesis	B
phoQ::xylE	gene	<i>phoQ</i>	PA1180	Mg ²⁺ sensing two-component sensor	B
23_B7	gene	<i>PA1291</i>	PA1291	Putative hydrolase	B
11_F7	gene	<i>PA2906</i>	PA2906	Probable oxidoreductase	B
12_D9	gene	<i>cifR</i>	PA2931	Cif transcriptional repressor	B
12_H2	gene	<i>nqrB</i>	PA2998	Na ⁺ -translocating NADH:ubiquinone oxidoreductase	A
20_B2	gene	<i>mmsB</i>	PA3569	3-hydroxyisobutyrate dehydrogenase	B
69_A6	gene	<i>PA3747</i>	PA3747	ABC-transport permease	B
76_D11	gene	<i>tgt</i>	PA3823	Queuine tRNA-ribosyltransferase	A
50_D9	gene	<i>pprB</i>	PA4296	Two-component response regulator	A, B
17_B9	gene	<i>PA4497</i>	PA4497	Binding protein component of ABC transporter	B
19_D2	gene	<i>pilV</i>	PA4551	Type 4 fimbrial biogenesis protein PilV	A
11_E1	gene	<i>PA4714</i>	PA4714	Predicted metal binding protein	B
12_F5	gene	<i>PA4936</i>	PA4936	Probable rRNA methylase	B
26_C3	gene	<i>PA4983</i>	PA4983	Two-component response regulator	A
80_B7	gene	<i>aceF</i>	PA5016	Dihydrolipoamide acetyltransferase	B
47_B5	gene	<i>typA</i>	PA5117	Regulatory GTPase	A
18_H10	gene	<i>dctD</i>	PA5166	Two-component response regulator	A
12_B5	gene	<i>gcvT1</i>	PA5215	Glycine-cleavage system protein T1	B
68_G8	gene	<i>PA5228</i>	PA5228	5-formyltetrahydrofolate cyclo-ligase	B
52_F4	gene	<i>cbcX</i>	PA5378	ABC-type choline transporter	B
12_H1	gene	<i>PA5472</i>	PA5472	ABC-type periplasmic transport protein	B
14_D8	gene	<i>PA5498</i>	PA5498	Probable adhesin	B

^a Refers to method A or B used as a secondary screen to confirm the preferred food source phenotype in Figure 3.