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Pseudomonas fluorescens NZI7 repels grazing by *C. elegans*, a natural predator

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Abstract

The bacteriovorous nematode *Caenorhabditis elegans* has been used to investigate many aspects of animal biology, including interactions with pathogenic bacteria. However, studies examining *C. elegans* interactions with bacteria isolated from environments in which it is found naturally are relatively scarce. *C. elegans* and bacteria from the *Pseudomonas fluorescens* species complex are frequently associated with cultivation of the edible mushroom *Agaricus bisporus*. We observed that pseudomonads isolated from mushroom farms showed differential resistance to nematode predation. Under nutrient poor conditions, in which most pseudomonads were consumed, the mushroom pathogenic isolate *P. fluorescens* NZI7 was able to repel *C. elegans* without causing nematode death. A draft genome sequence of NZI7 showed it to be closely related to the biocontrol strain *P. fluorescens* Pf-5. To identify the genetic basis of nematode repellence in NZI7, we developed a grid-based screen for mutants that lacked the ability to repel *C. elegans*. The mutants isolated in this screen included strains with insertions in the global regulator GacS and in a previously undescribed GacS-regulated gene cluster, 'EDB' ("edible"). However, heterologous expression of the EDB gene cluster in other pseudomonads did not repel nematode feeding. This suggests that the product(s) of the EDB locus act additively or synergistically with other features of NZI7 to provide a novel mechanism to repel nematode grazing.

Keywords

Brown blotch disease / chemotaxis / dehydroquinate synthase (DHQS) / *Pseudomonas tolaasii* / cyanide / tolaasin

Introduction

Laboratory studies of bacteria-nematode interactions, most notably studies using clinical isolates of *Pseudomonas aeruginosa*, have shown that many environmental and human pathogenic bacteria possess mechanisms to inhibit bacteriovores such as *C. elegans* (Bjornlund et al 2009, Irazoqui et al 2010, Niu et al 2010, O'Quinn et al 2001, Pedersen et al 2009, Powell and Ausubel 2008, Rae et al 2010, Sifri et al 2005, Tampakakis et al 2009, Tan et al 1999, Troemel et al 2008, Zaborin et al 2009). Examples of such mechanisms include poisoning by cyanide production (Gallagher and Manoil 2001), pore formation by *Bacillus thuringiensis* Cry toxins (Marroquin et al 2000) and biofilm formation across the nematode pharynx by *Yersinia pestis* (Darby et al 2002). Aversive olfactory responses to bacteria have also been described (Ha et al 2010, Pradel et al 2007, Shtonda and Avery 2006, Zhang et al 2005). However, studies examining the interaction of *C. elegans* with the bacteria it encounters in natural environments are relatively scarce (Freyth et al 2010; Félix and Braendle 2010).

The model strain *C. elegans* strain N2 was originally isolated from a mushroom farm (Chen et al 2006, Grewal and Richardson 1991, Hansen et al 1959). *C. elegans* has also been isolated from compost, snails and rotting fruit (Barriere and Felix 2005, Barriere and Felix 2007, Caswell-Chen et al 2005). On mushroom farms *C. elegans* is known to colonise the fruiting bodies (sporophores) of *Agaricus bisporus,* where bacteria belonging to the genus *Pseudomonas* commonly occur (Grewal 1991a, Grewal and Richardson 1991). Synergistic interactions between *C. elegans* and mushroom pathogenic pseudomonads have been reported to increase the severity of blotch disease of cultivated mushrooms (Grewal 1991a, Grewal 1991b). Furthermore, fluorescent pseudomonads both antagonistic and beneficial to mushroom growth have been isolated directly from *C. elegans* (Grewal 1991a, Grewal and Hand 1992). Thus in natural environments, *C. elegans* are likely to

interact more frequently with pseudomonads than with bacteria that are commonly used as food sources in laboratory studies, such as *Escherichia coli* (Atkey et al 1992, Curran et al 2005, Grewal and Hand 1992, Tsukamoto et al 2002, Viji et al 2003, Zarkower et al 1984). We have found that many pseudomonads isolated from mushrooms possess mechanisms for inhibiting nematode growth or deterring nematode feeding. Our investigation of the mushroom pathogenic pseudomonad *P. fluorescens* NZI7, described here, has uncovered a novel and highly effective mechanism that deters nematode feeding, even in nutrient limited conditions where production of nematicidal factors is insufficient to confer protection.

Materials and Methods

Bacteria and nematode cultivation

Pseudomonas and *E. coli* strains were cultured on Luria-Bertani (LB) medium (Sambrook and Russell 2001) at 28ºC and 37ºC respectively for 24 hours prior to use. Strains used are listed in Table S1. Wild-type *Caenorhabditis elegans* Bristol N2 and *Caenorhabditis briggsae* nematodes were maintained at either 15ºC, 20ºC or 22ºC on nematode growth medium (NGM) (Brenner 1974) inoculated with *E. coli* OP50 and synchronous cultures were produced according to the protocols available on Nematodebook (Girard et al 2007). Antibiotics were used at the following concentrations: kanamycin 50 µg/ml, tetracycline 20 µg/ml, chloramphenicol 25 µg/ml. Nematode rapid killing assays were performed on brain heart infusion (BHI) agar (Oxoid).

Quantitative nematode feeding and choice assays

Quantitative nematode feeding and choice assays were performed by washing overnight bacterial cultures twice in dH_2O and resuspending bacteria at an OD_{600} of 0.1. Fifty microlitres of this suspension were spotted onto the centre of a 45 mm diameter NGM plate and incubated for 24 hours at 28ºC. Ten synchronous L2/L3 nematodes were transferred to each plate and plates were incubated at 20ºC. Lawns were photographed daily and the area occupied by the lawn determined using the magnetic lasso function in Adobe Photoshop (Adobe Systems Incorporated, San Jose). Mixed bacterial populations were tested similarly, except that overnight cultures were mixed in different ratios prior to aliquoting onto NGM.

Choice assays were set up by spotting 25 µl of an overnight culture of the strains to be tested equidistant from a central point on an NGM plate and incubating the plates for two days at 28ºC. Nematode preference was assessed by placing approximately 20 L3/L4 nematodes into the centre of each plate and scoring for presence of nematodes and the extent to which each colony was consumed over 3 days at 22 ºC. Chemotaxis assays were performed similarly except larger numbers of nematodes were used per plate (~200) and the number of nematodes at the bacterial colonies counted six hours after transfer. The chemotaxis index was calculated as (number on spot 2 - number on spot 1)/ total number of nematodes at both spots.

Biochemical complementation of edible mutants was tested by supplementing NGM plates with 1 mM thiamine or 1 mM anthranilic acid. Plates streaked with mutants of interest were incubated for 24 hours at 28ºC and then between 15 and 20 L3/L4 nematodes were transferred to the centre of the lawns. Plates were incubated at 20ºC and observed daily for 5 days.

Screen for loss of nematode repellence

The construction and phenotypic analysis of an ordered library of 9696 insertional mutants of NZI7 using miniTn5::*gfp::luxABCDE* is described in Supplementary Methods. Oligonucleotides used are listed in Table S2. To identify mutants that showed reduced ability to repel nematodes, mutants were replicated using a 48-point colony replicator onto NGM plates. Two colonies of *E. coli* OP50 were inoculated at opposite sides of the mutant grid to serve as a food source while nematodes explored the plate. Plates were incubated for two days at 28ºC to allow colonies to grow. After incubation, approximately 20 L4/adult nematodes were placed adjacent to the *E. coli* colonies. Plates were incubated for 3 days at 20ºC and observed daily. The mutant library was screened twice using this protocol. Mutants identified in the initial screen were validated in two replicate 'dummy grids', in which 46 of the positions contained wild-type NZI7, and as individual lawns. Individual edibility assays were performed by streaking the test strain across an NGM or BHI plate, allowing the strain to grow for 24 hours and then placing approximately 20 synchronous L2/L3 nematodes in the centre of the bacterial lawn.

Results

P. fluorescens NZI7 repels feeding by *C. elegans*

We tested a collection of 60 *Pseudomonas* isolates, including 32 strains isolated from mushroom farms, to determine whether they showed differential interactions with *C. elegans*. Considerable heterogeneity in nematode-bacteria interactions was observed; some strains showed high resistance to nematode grazing, while others supported higher rates of nematode growth and reproduction than *E. coli* OP50, which is commonly used as a food source for *C. elegans* (Table 1). One strain, the mushroom pathogen *P. fluorescens* NZI7 (Godfrey et al 2001b; henceforth referred to as NZI7), was of particular interest, as it exhibited a distinctive and highly effective

mechanism for repelling *C. elegans* without killing when grown on NGM. The same phenomenon was observed with the related species *Caenorhabditis briggsae* (data not shown). Nematodes initially explored NZI7 lawns but became averse to further interaction, preferentially occupying bacteria-free parts of the agar surface and showing a severe impairment in growth. When the assay was repeated using nutrient rich BHI media, nematodes died after a few hours exposure to NZI7. We quantitatively analysed nematode feeding by monitoring the area of the bacterial lawn and nematode size. Both NZI7 and the biocontrol strain *P. fluorescens* Pf-5 (henceforth referred to as Pf-5), were highly resistant to nematode grazing (Figure 1). Pf-5 produces an arsenal of molecules with anti-eukaryote activity that could target *C. elegans* (Loper and Gross, 2007), and caused nematode death on both BHI and NGM.

Chemotaxis mutants of *C. elegans* are able to feed on NZI7 without exhibiting deleterious effects

In binary choice assays in which nematodes were able to choose between colonies of *E. coli* OP50 or NZI7, wild-type nematodes were strongly repelled by NZI7 (chemotaxis index -0.91 ± 0.09, N = 6). In contrast, *C. elegans* chemotaxis mutants *tax-2* and *tax-4* (reviewed by Bargmann 2006) showed substantially reduced repulsion (chemotaxis indices -0.38 ± 0.18, N=5 and –0.44 ± 0.15, N =7, respectively). Complete loss of repulsion would result in a chemotaxis index of 0, so the residual repulsion suggests that avoidance of NZI7 has both chemotactic and non-chemotactic elements. Recent work by Chang et al (2011) has demonstrated that pathogen avoidance by *C. elegans* can be mediated by the lateral outer labial (OLL) mechanosensory head neurons. *tax* mutants feeding on NZI7 grew well and showed no evidence of deleterious effects or pharyngeal dysfunction, allowing us to

exclude the possibility that failure of nematodes to feed was due to a physical effect, as reported in interactions of nematodes with *Y. pestis* (Darby et al 2002). The resistance of NZI7 to nematodes was stable with no evidence of either nematode lethality or later nematode acclimation.

The *P. fluorescens* NZI7 genome is similar to that of the biocontrol strain *P. fluorescens* Pf-5

NZI7 causes brown blotch disease on mushrooms, which was attributed to production of a lipodepsipeptide similar to tolaasin (Godfrey et al 2001a). Phylogenetic analysis of two highly conserved housekeeping genes revealed that although NZI7 produces a diffusible molecule that behaves like tolaasin in the white line bioassay (WLA) it is more closely related to *P. fluorescens* Pf-5 than to the tolaasin-producing type strain *P. tolaasii* NCPPB 2192 (Figure S1). To identify candidate genes involved in NZI7-nematode interactions we generated a draft NZI7 genome (see Supplementary methods). *De novo* assembly of 165,948 reads (~8x coverage) generated 1034 contigs with a sum of contig lengths of 6,814,598 nt. The genome of NZI7 showed a high degree of similarity to that of Pf-5, consistent with their close phylogenetic relationship (Figure S2).

To identify regions of nucleotide sequence similarity between Pf-5 and NZI7, we used BLASTN searches with an E-value threshold of 10^{-6} between the Pf-5 genome (Paulsen et al 2005) and NZI7 genome sequence data (both the *de novo* assembly and the raw sequence reads). The genome of Pf-5 contained 972 predicted genes that showed no detectable nucleotide sequence similarity with NZI7 (Table S3). NZI7 lacks several gene clusters previously described in Pf-5 including those involved in the production of rhizoxin, the Mcf/Fit toxin, pyoluteorin, orfamide and pyrrolnitrin, allowing us to discount these as being responsible for the repellence and lethality of

NZI7 to nematodes when grown on NGM and BHI respectively. The NZI7 genome does contain genes predicted to be involved in synthesis of cyanide, 2,4-diacetylphloroglucinol (DAPG) and the extracellular protease AprA, all of which have been implicated in inhibition of *C. elegans* or of plant parasitic nematodes such as *Meloidogyne incognita* (Gallagher and Manoil 2001, Meyer et al 2009, Neidig et al 2011, Siddiqui et al 2005). However, one recent study has shown that purified DAPG promotes egg hatch in *C. elegans* J1, and has no effect on the viability of juvenile or adult nematodes (Meyer et al 2009).

The majority of the 602 genes detected in NZI7 and not Pf-5 (Table S4) are not obviously associated with nematode repellence or toxicity. Therefore the ability of NZI7 to repel *C. elegans* may be a property encoded by the shared gene complement of NZI7 and Pf-5, which is masked in Pf-5 by the presence of additional nematicidal factors. However, the NZI7 genome is predicted to encode a number of cell surface-associated genes that are not present in Pf-5, including genes associated with exopolysaccharide synthesis, type IV pilus and fimbrial assembly and type II, IV, V and VI secretion. Cell surface structures have been shown to influence bacterial interactions with nematodes (e.g. Essex-Lopresti et al 2005; Maier et al 2010). Thus, it is possible that NZI7-specific factors contribute to repellence.

Identification of 'edible' mutants of *P. fluorescens* NZI7

To identify the genetic basis of nematode repellence in NZI7 we constructed an ordered library of 9696 insertional mutants using a mini-Tn5 transposon that contains a promoterless *gfp*::*luxABCDE* reporter cartridge (Fones et al 2010). We developed a grid-based assay to identify mutants that were unable to repel *C. elegans* (Figure 2), which was validated by confirming that nematodes were able to locate "edible" pseudomonads, such as *P. fluorescens* SBW25, placed in random positions in a grid

205 of wild-type NZI7 colonies (data not shown). The library was also used to identify
206 mutants with alterations in other traits that might affect mushroom pathogenesis or
207 nematode predation: lipase, chitinase and lecithinase activity, cyanide production,
208 haemolysis and production of the tolaasin-like toxin (TOL) (Table S6). Transposon
209 insertion points were determined by two step semi-degenerate PCR (Jacobs et al
210 2003).

211 A total of 84 NZI7 transposon mutants (0.87% of the total, summarized in Table 2)
212 were identified and validated as “edible”. Of these 84 mutants, 45 displayed a wild-
213 type phenotype in other screens performed on the mutant library. Sequencing led to
214 the determination of insertion points for 75 mutants. Overlay of transposon hits onto
215 corresponding ORF positions in the draft NZI7 genome, mapped onto the complete
216 genome of Pf-5, showed that none of these mutants were unique to NZI7 (Figure
217 S2). However, eleven “edible” mutants that had wild-type phenotypes in the other
218 screens performed were associated with a single gene cluster of unknown function,
219 present in both NZI7 and Pf-5 (Figure 3), which is henceforth referred to as the EDB
220 (edible) gene cluster. The complete NZI7 EDB gene cluster (EDB ORFs 1-12) was
221 sub-cloned into the cosmid pLAFR6 to create pLAF4EDB and transformed into NZI7
222 EDB mutants. The presence of pLAF4EDB restored the ability of EDB cluster
223 mutants to repel *C. elegans*, confirming the role of the EDB cluster in nematode
224 repellence (Figure 4).

225 Several of the “edible” mutants identified in the screen were found have insertions in
226 genes previously shown to be important in *P. aeruginosa* pathogenesis towards *C.*
227 *elegans*: the global virulence regulator *gacS*; *algU,* a negative regulator of alginate
228 biosynthesis, which was associated with a mucoid colony morphology; *dsbA,*
229 required for correct folding of periplasmic disulphide bonded proteins, the
230 phosphoenolpyruvate-protein phosphotransferase gene *ptsP*, purine biosynthesis
231 genes; the two-component sensor *cbrA;* and a haemagglutinin repeat protein with

232 weak similarity to PA0041 (Gallagher and Manoil 2001, Reddy et al 2011, Tan et al
233 1999, Tan 2002, Yorgey et al 2001). Edible mutants with insertions in genes with
234 predicted roles in aromatic amino acid metabolism (anthranilate and chorismate
235 synthase genes) and thiamine biosynthesis could be chemically complemented by
236 addition of 1 mM anthranilate or thiamine to the growth medium respectively (Figure
237 S3).

238 *C. elegans* has been shown to avoid *Serratia marcescens* Db10 producing the
239 lipodepsipeptide serrawettin W2 (Pradel et al 2007). However, NZI7 mutants
240 disrupted in the biosynthesis of TOL retained the ability to repel *C. elegans*. TOL
241 mutants were, however, unable to cause disease symptoms on mushroom
242 sporophore tissue (Figure S4), confirming the importance of this toxin for mushroom
243 pathogenesis. In contrast, mutation of the EDB cluster did not alter disease
244 symptoms on mushroom tissue.

245

246 **Delineation and bioinformatic analysis of the EDB gene cluster**

247 The EDB gene cluster is highly conserved in both NZI7 and Pf-5 (Figure 3). EDB-like
248 gene clusters were also detected in the genomes of several other pseudomonads: *P.*
249 *fluorescens* WH6 (Kimbrel et al 2010), *Pseudomonas brassicacearum* NFM421
250 (Ortet et al 2011), *P. brassicacearum* Q8r1-96 (Loper et al 2012), and the insect
251 pathogen *Pseudomonas entomophila* L48 (Vodovar et al 2006b). We have also
252 identified EDB-like gene clusters in the draft genomes of other mushroom pathogenic
253 pseudomonads, including *P. fluorescens* NZ007, *P. tolaasii* NCPPB2192, *P. tolaasii*
254 PMS117S and *P. gingeri* NCPPB3146 (Figure S5). The genomic context of the EDB
255 cluster is not conserved in all of these strains, but four additional ORFs found
256 downstream of the NZI7 EDB cluster genes, corresponding to Pfl_5548-Pfl_5551 in
257 Pf-5, can be found adjacent to the EDB-like genes in all but two of these strains

(Figure S5). Two of these ORFs, ORF 10 (Pfl_5549) and ORF 11 (Pfl_5550), display some similarity to ORF 7 (Pfl_5546) and ORF 8 (Pfl_5547) respectively. We were unable to detect features characteristic of genomic islands associated with the EDB region. However, a partial transposase-like sequence is located adjacent to the EDB-like gene cluster in *P. fluorescens* WH6.

To test whether these four downstream ORFs were involved in nematode repellence, and to further explore the functions of ORFs within the EDB cluster we constructed deletion mutants lacking ORF 1, ORFs 9-12 and the complete cluster (ORFs 1-12). Mutants lacking ORF1 or the complete cluster displayed a similar loss of repellence to the transposon mutants identified in the initial screen. However, mutants lacking ORFs 9-12 retained the ability to repel nematode predation (data not shown). Thus, only ORFs 1-8, corresponding to Pfl_5540-Pfl_5547 in Pf-5, are required for nematode repellence.

The majority of the 12 ORFs in the EDB cluster show only weak sequence similarity to genes with known or predicted functions (Table 2, Table 3). However, the presence of a 3-dehydroquinate synthase (DHQS) domain in the first ORF, a prenyltransferase family domain in the second ORF, and the similarity of additional ORFs to enzymes involved in modification of carbohydrate substrates, suggests that this cluster encodes enzymes that contribute to the synthesis of a novel compound. Functional predictions for EDB cluster proteins, based on structure prediction using I-TASSER (Roy et al 2010), also suggest that they are involved in the synthesis or modification of a carbohydrate-containing compound (Table 3).

Bioinformatic analyses using PSORTdb V2.0 (http://www.psort.org/psortb/ (Rey *et al.*, 2005) and Phobius (http://phobius.sbc.su.se/ (Kall *et al.*, 2004) indicated that the putative DHQS-like and prenyltransferase EDB proteins are likely to be membrane associated while the remaining proteins are predicted to be cytoplasmic. The domain

architecture of the first ORF is atypical for DHQS proteins since it contains a domain similar to the SNARE-associated (SNARE_assoc) superfamily domain at the N-terminus. This suggests that some of the proteins encoded in the EDB cluster are associated with the membrane or cell surface. We speculate that the compound(s) synthesized by these proteins may also remain membrane or cell surface associated, which is consistent with the observation that nematodes can discriminate between colonies grown in close proximity in the grid screen.

C. elegans grows most rapidly on _gacS_ mutants of _P. fluorescens_ NZI7

Interestingly, although results from the mutant screen suggested that the product of the EDB locus was likely to be a primary cause of nematode repellence, *gacS* mutant lawns were consumed more rapidly than other mutants, including EDB cluster mutants, and supported faster nematode growth rates (Figure 4C and 4D). Furthermore, nematodes were less repelled by the *gacS* mutant than other mutants isolated in the EDB screen (Figure 5). GacS is a global regulator that is required for the expression of numerous virulence and antagonism-related genes in other pseudomonads (Blumer et al 1999, Lapouge et al 2007, Rahme et al 1995, Vodovar et al 2006a). Consistent with this, NZI7 *gacS* mutants gave negative results in TOL, chitinase, lipase, lecithinase and cyanide assays (Table S5). However, although we were able to identify mutants that lacked the ability to produce each of these individual factors (Table S5), they all retained the ability to repel nematode predation, as did deletion mutants lacking the DAPG biosynthetic cluster (data not shown).

GacS regulates expression of the EDB gene cluster

The promoterless *luxABCDE* reporter cassette in the mini-Tn5 transposon provides a tool for examining gene expression when the transposon is oriented to place this cassette under the control of an endogenous promoter. We tested whether GacS regulated the expression of the EDB cluster by deleting *gacS* in two such mutants (79G1 and 54G10), in which the transposon is inserted into ORF 1 (PFL_5540) and ORF 8 (PFL_5547) respectively. Luminescence was strongly reduced in double ΔGacS/EDB mutants relative to the EDB reporter mutants (Figure 6A). We observed a similar reduction in luminescence when *gacS* was deleted in mutant 5E3, in which the transposon is inserted in the TOL biosynthetic locus (Figure 6B, Table S5). Luminescence in *ΔgacS*-EDB reporter mutants could be restored to wild-type levels by complementation with *gacS* (Figure 6C).

RT-PCR experiments using conditions favouring EDB expression indicated that EDB cluster mRNA was absent in NZI7 *gacS* mutants (Figure 6D) raising the possibility that GacS may regulate EDB at a transcriptional level rather than at the post-transcriptional level described for several other GacS-regulated genes (Heeb et al 2005, Kay et al 2005, Whistler et al 1998). However, we cannot exclude the possibility that the lack of EDB mRNA reflects RNA instability rather than transcriptional regulation (Lapouge *et al.* 2008). Interestingly, global transcriptional analysis of Gac-regulated genes in Pf-5 did not identify PFL_5540-PFL_5551 as components of its Gac regulon (Hassan et al 2010), suggesting that the regulation of this locus may differ between the two strains.

Environmental regulation of EDB expression

We used the *lux* reporter fusions within the EDB cluster to monitor the impact of environmental conditions on EDB gene expression. EDB expression was enhanced by mildly acidic pH, rich media such as KB, moderate-high iron availability and an

optimal carbon-nitrogen balance (Figure S6A-D). EDB expression increased in late log and early stationary phase but was repressed in stationary phase by carbon sources such as glucose and mannose (Figure S6E). Expression was not significantly altered by introduction of the complementing clone pLAF4EDB into reporter strains, indicating that it is not autoregulatory (Figure S6F). Since EDB mutants continue to produce TOL, cyanide and exoenzymes (Table S5) the product of the EDB cluster does not seem to affect the expression of other GacS-regulated factors.

The product(s) of the EDB gene cluster act with other factors to deter nematode feeding

The success of the grid screen in identifying edible mutants suggests that the factor(s) produced by the EDB locus are not active as a diffusible signal over distances greater than 5 mm, since the phenotype of EDB mutants would be masked by surrounding colonies if this were the case. *gacS* mutants of *P. fluorescens* CHA0 have previously been shown to be protected by wild-type bacteria from *C. elegans* feeding when present at low frequency in mixed populations (Jousset et al 2009). When we fed *C. elegans* mixtures of NZI7 wild-type and EDB cluster mutants in different ratios grown on NGM, nematodes would clear lawns where the initial ratio of EDB mutant: wild-type was (5:1 or greater). This suggests that nematodes make a decision based on an aggregate assessment or that there is a critical level at which the putative EDB product acts, below which it becomes ineffective.

We transformed several palatable *P. fluorescens* isolates with the cloned NZI7 EDB gene cluster (pLAF4EDB) and were able confirm EDB expression in most of the transformed strains by RT-PCR (Figure S7). However, none of the transformed strains showed enhanced resistance to *C. elegans*, either on NGM or on BHI medium

on which EDB is more strongly expressed (Figure S6B). *E. coli* transformed with pLAF4EDB also failed to recapitulate the repellent phenotype, although EDB expression was comparatively poor in this background (data not shown). It thus appears that EDB alone is insufficient to deter nematode feeding, which is consistent with the presence of the EDB gene cluster in pseudomonads that do not show strong nematode repellence.

Cyanide is responsible for rapid nematode killing by NZI7

One factor that could act in conjunction with EDB to repel nematode predation is cyanide, which has been implicated in nematode repellence in *P. fluorescens* CHA0 (Neidig et al 2011). NZI7 produces high levels of cyanide on BHI, causing rapid nematode killing by lethal paralysis, as previously reported for *P. aeruginosa* PAO1 (Gallagher and Manoil 2001). However, NZI7 did not produce detectable levels of cyanide when grown on NGM and a non-cyanogenic *hcnB* mutant was indistinguishable from the wild-type in nematode repellence assays on NGM. On BHI the *hcnB* mutant failed to kill nematodes but was highly repellent. An *hcnB*/EDB double mutant showed significantly less repellent activity than the *hcnB* mutant on BHI; some nematodes remained on the bacterial lawn and surrounding agar and grew to produce eggs within three days (Figure S9). This confirmed that EDB contributes to nematode repellent activity on BHI and that cyanide is the primary nematicidal toxin produced by NZI7 on BHI. However, NZI7 *gacS* mutants supported a much larger nematode population than the *hcnB*/EDB mutant on BHI, which supports the hypothesis that additional *gacS*-regulated factors contribute to the inhibition of nematode growth. TOL does not appear to be one of these factors, since a triple *hcnB*/EDB/TOL mutant was indistinguishable from the *hcnB*/EDB double mutant on BHI and NGM (data not shown).

Discussion

The results presented in this study show that the mushroom pathogen *P. fluorescens* NZI7 is able to repel grazing by *C. elegans*, a naturally occurring bacteriovore in mushroom farms. The ability of NZI7 to repel *C. elegans* depends on the activity of a previously uncharacterised biosynthetic locus, the EDB cluster. Interestingly, EDB-dependent nematode repellence is effective even in low nutrient environments, where nematicidal factors such as cyanide are ineffective at limiting nematode predation. As the product of the EDB cluster seems to have no effect on its own expression, or on the expression of other GacS-regulated genes, it is logical to hypothesise that the product(s) of this cluster is sensed directly by *C. elegans*. However, heterologous expression of the EDB gene cluster in several palatable pseudomonads failed to recapitulate the phenotype seen in NZI7. Thus, while the EDB cluster is important for nematode repellence in NZI7, additional loci are necessary for NZI7 to effectively deter nematode predation.

The observation that EDB mutants of NZI7 retain some ability to repel and inhibit nematodes relative to *gacS* mutants raises the possibility that the product of the EDB locus acts additively or synergistically with other factors to reach a threshold at which *C. elegans* decides to avoid grazing. We identified 40 distinct mutations, other than those within the EDB cluster, which reduced nematode repellence in NZI7. Some of these may be involved in the synthesis of precursor molecules needed for the synthesis of the EDB product, while others, such as *algU*, may cause changes in exopolysaccharide production that compromise the ability of nematodes to respond to bacterial signals (Reddy et al 2011). However, others could be involved in the synthesis of a second nematode repellent factor. It is also possible that mutations affecting additional nematode repellent factors may not have been detected in the

412 grid screen if these factors are highly diffusible or individually contribute only a small
413 fraction of the total NZI7 repellent activity.

414 *C. elegans* has been shown to respond to a wide range of chemoattractants and
415 repellents (Bargmann 2005, Dusenbery 1975, Hilliard et al 2004, Ward 1973) and
416 there is emerging evidence for the perception of bacterial metabolites by nematodes,
417 notably quorum sensing molecules and serrawettin W2 (Beale et al 2006, Pradel et al
418 2007). Thus, the EDB locus may be involved in the synthesis of a molecule that
419 identifies NZI7 as a strain to be avoided, even though the product of the EDB cluster
420 itself is not directly toxic to nematodes. In this case the inability of strains
421 heterologously expressing the EDB cluster to repel nematode predation could be due
422 to the absence of additional loci required for synthesis of the EDB product, or the
423 absence of additional factors that act together with EDB to deter nematode grazing.
424 The ability to preferentially feed on non-deleterious bacteria would clearly be
425 advantageous to nematodes in the natural environment, where bacterial populations
426 are heterogeneous (Laws et al 2006, Rodger et al 2004, Shtonda and Avery 2006,
427 Zhang et al 2005).

428 The grazing resistance of NZI7 is particularly intriguing when viewed in terms of the
429 mushroom pathogenic lifestyle of this bacterium. We have observed that nematodes
430 investigate bacterial colonies and then move away without consuming large
431 quantities of bacteria rather than completely avoiding them. This physical contact
432 between nematodes and NZI7 means that bacteria become transiently associated
433 with the surface of the nematode, and in our assays NZI7 is clearly transported by
434 the nematodes, visible as bacterial trails from visited colonies. In a natural setting,
435 such as a mushroom farm, the ability of NZI7 to be disseminated by nematodes,
436 while avoiding nematode predation, could enhance both the survival and dispersal of
437 this mushroom pathogen, contributing to the development of blotch disease.

438

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Table 1: Growth and behaviour of *Caenorhabditis elegans* on lawns of *Pseudomonas* cultivated on nematode growth medium (NGM)

	Strain	Rank[a]	Observations (0-72 hours)
P.fl[b]	**NZ047**[c]	1+	Egg laying at <44 hours
P.fl	WCS365	1+	Egg laying at <44 hours

P.fl	**NZ062**	1+	Egg laying at <44 hours
P.fl	**NZ113**	1+	Egg laying at <44 hours
P.to	**NCPPB 2192**	1+	Egg laying at <44 hours
P.fl	**NZ009**	1+	Egg laying at <44 hours, long
P.fl	**NZ43**	1+	Egg laying at <44 hours
P.fl	**NZ092**	1	Egg laying within 46 hours
P.sy	DC3000	1	Egg laying within 46 hours
P.ae	**NZ017**	1	Egg laying within 46 hours
P.sy	B728a	1	Egg laying within 46 hours
P.sy	1448A	1	Egg laying within 46 hours
P.vi	PC006	1	Egg laying within 48 hours
P.en	L48	2	Egg laying within 48 hours, similar to OP50
P.fl	**NZ065**	2	Egg laying within 48 hours
P.sy	PN2	2	Egg laying within 48 hours
P.sy	870A	2	Egg laying within 48 hours
P.fl	**NZ102**	2	Egg laying within 48 hours
P.fl	**NZ043**	2	Egg laying within 48 hours
P.fl	**NZ096**[d]	2	Egg laying within 48 hours
P.fl	**NZ060**	2	Egg laying within 48 hours
P.gi	**NCPPB3146**	2	Egg laying within 48 hours
P.fl	WH6	2	Egg laying within 48 hours
P.fl	SBW25	2	Egg laying within 48 hours
P.fl	**NZ024**	2	Egg laying within 48 hours
P.fl	**NZ014**	2	Egg laying within 48 hours
P.fl	**NZ104**[d]	2	Egg laying within 48 hours
P.fl	EJP115[d]	3	Some growth delay
P.re	**NCPPB 387**	3	Some growth delay, no eggs after 48 hours

P.fl	WCS417	3	Variable sizes, few eggs, fertility inhibited
P.fl	OE28.3	3	Many adults after 48 hours
P.fl	**NZ112**[d]	3	Some eggs at 48 hours, many adults, sluggish
P.sy	B301D	3	Egg laying within 48 hours, loopy
P.fl	**NZ006**	3	Egg laying within 50 hours
P.fl	**NZ103**[d]	3	Egg laying within 50 hours
P.fl	**NZ031**	3	Egg laying within 50 hours, thin, uncoordinated
P.fl	**NZ011**[d]	3	Egg laying within 50 hours, thin
P.fl	**NZ007**	3	Egg laying within 50 hours, thin
P.fl	BBc6R8	3	Some growth delay, lawn well grazed[e]
P.fl	EJP116[d]	4	Some growth delay, small adults
P.fl	**NZ039**[d]	4	Variable sizes, thin, a few eggs
P.fl	Pf0-1	4	Variable sizes, a few eggs
P.fl	**NZ111**	4	Variable sizes, a few eggs
P.pu	WCS358r	4	Egg hatch inhibited, egg Egg laying inhibited
P.pu	UWC1	4	Growth moderately inhibited, some adults present
P.sy	61	4	Growth moderately inhibited, thin
P.fl	**NZ052**	5	Growth moderately inhibited, lawn ungrazed
P.fl	WCS374r	5	Growth moderately inhibited, low fertility, lawn ungrazed
P.to	**PMS117S**	5	Growth moderately inhibited, very few adults, lawn ungrazed
P.ae	PA14	5	Growth moderately inhibited
P.fl	**NZ124**	6	Growth moderately-strongly inhibited, small nematodes, no eggs
P.fl	**NZ059**	6	Growth strongly inhibited
P.fl	F113	6	Growth strongly inhibited
P.ae	PA01	6	Growth strongly inhibited

P.fl	**NZI7[d]**	7	Growth strongly inhibited, lawn ungrazed
P.ch	PCL1391[d]	7	Growth strongly inhibited, lawn ungrazed
P.fl	**NZ101[d]**	8	Dead within hours, tracks visible
P.fl	Pf-5[d]	8	Dead within hours, few tracks
P.fl	**NZ097[d]**	8	Dead
P.ma	CTA23	8	Dead

749

750 [a] The rank of each strain was assigned according to the key below; [b] species name
751 abbreviations are *P.fl, Pseudomonas fluorescens, P.to, Pseudomonas tolaasii, P.sy,*
752 *Pseudomonas syringae, P.vi, Pseudomonas viridiflava, P.ae, Pseudomonas*
753 *aeruginosa, P.en, Pseudomonas entomophila, P.re, Pseudomonas reactans*; *P.gi,*
754 *Pseudomonas gingeri*; [c] bold font indicates that a strain was isolated from a
755 mushroom farm; [d] indicates that the strain was observed to cause rapid killing on
756 brain heart infusion (BHI) agar; [e] grazed/ungrazed refers to the extent to which the
757 bacterial lawn was visibly depleted over time through nematode predation.

758

Rank	Phenotype
1+	Eggs produced within 48 hours, lawn grazed quicker than OP50
1	Eggs layed by 48 hours, lawn grazing similar to OP50
2	Some eggs laid by 48 hours, development slower than on OP50
3	Longer delay in development, eggs produced between 48 and 72 hours
4	Significant delay in development, few eggs after 72 hours,
5	Moderate inhibition of growth, lawn largely ungrazed
6	Nematodes strongly inhibited, few mostly small nematodes, few or no eggs
7	Nematodes strongly inhibited, lawn ungrazed, no egg production
8	Nematodes dead within hours

759

760 **Table 2** NZI7 transposon mutants that show reduced nematode repellence relative to
761 wild-type bacteria

Mutant[1]	Identity	Best hit[2]	Rep[3]
7F10*	Thiol:disulfide interchange protein, DsbA family	PFL_0085	-
7B2, 7F4	Glutaryl-CoA dehydrogenase	PFL_0117	+
7C10, 17G3*	Glutamate-cysteine ligase	PFL_0273	-
16A11	Histidinol dehydrogenase	PFL_0929	+
84H8*	MucA sigma factor AlgU	PFL_1449	-
9D1*	Intracellular septation protein A	PFL_1596	+
20H10*	Cytochrome c-type biogenesis protein CycL	PFL_1685	-
17G1	Glyoxylate carboligase	PFL_1701	+
15D2	3-isopropylmalate dehydrogenase	PFL_2066	+
19G12, 95A6*	Intergenic: conserved hypothetical protein/pentapeptide repeat family protein	PFL_2214/2215	-/+
85A12	Haemagluttinin repeat protein	PFL_2761	+
32F2, 28H5*	Predicted transcription regulator containing HTH domain	PFL_2816	-
7A5	NADH-quinone oxidoreductase, G subunit	PFL_3902	+
20E8*	OmpA porin-like integral membrane protein	PFL_3930	+
10H11	ATP-dependent Clp protease	PFL_3987	+
55F9	Periplasmic binding protein	PFL_4192	+
25G4	Integration host factor	PFWH6_1610[4]	+

39G12, 91G1, 74A9*	Chorismate synthase	PFL_4348	+
93C1	(p)ppGpp synthetase I (GTP pyrophosphokinase)	PFL_4446	+
33D6	Cysteine synthase B	PFL_4448	-
35A8, 21A6, 21C6*	GacS sensor kinase protein	PFL_4451	+
52C3, 55C2, 52A7*	Glycerol-3-phosphate dehydrogenase, FAD-dependent	PFL_4870	+/-
48A1	Acetolactate synthase, large subunit	PFL_5255	+
3A4*	Sensory box histidine kinase	PFL_5273	-
94B1	3-methyl-2-oxobutanoate hydroxymethyltransferase	PFL_5277	+
50A5	Glutamate 5-kinase	PFL_5326	-
23G12*	Major facilitator family transporter	PFL_5388	-
82A10	FadE8, putative acyl-CoA dehydrogenase	PFL_5425	+
101B8, 79G1, 15G6, 79D6	**3-dehydroquinate synthase domain protein**	**PFL_5540**	**-/+**
50G3	**UbiA prenyltransferase family superfamily**	**PFL_5541**	**-**
14E12, 99G1	**Hypothetical protein**	**PFL_5542**	**+**
90E9, 98H10	**Hypothetical protein**	**PFL_5544**	**+**
7A7	**Conserved hypothetical protein**	**PFL_5545**	**-**
54G10	**Hypothetical protein**	**PFL_5547**	**+**
50C11, 89C9	Anthranilate synthase component I	PFL_5629	-
21C10*	Bis(5'-nucleosyl)-tetraphosphatase	PFL_5651	-

95C3*	Zn-dependent dipeptidase	PFL_5742	-
11G5	Thiazole biosynthesis protein ThiG	PFL_5850	-
90D7	Dihydroxy-acid dehydratase	PFL_5877	+
14F7	Phosphoenolpyruvate-protein phosphotransferase PtsP	PFL_5899	-
66C3*	Dinucleoside polyphosphate hydrolase	PFL_5900	+
77C3, 82A12*	Penicillin amidase family protein	PFL_5919	-
64H11, 25A7	Phosphoribosylaminoimidazole carboxylase, ATPase subunit	PFL_6125	+/-
31H3, 75C2, 86B12, 93G12, 94D11	Acetyl-CoA carboxylase, biotin carboxylase	PFL_6158	+/-
33H7, 8D5, 37A1, 46H6,	Glucose inhibited cell division protein *gidA*	PFL_6226	-/+
14G6, 83F10, 16G5, 43D3* 6H10*	tRNA modification GTPase *trmE*	PFL_6229	+
53A4*	Inner membrane protein, 60 kDa	PFL_6230	-

762

763 [1]Mutants or sets of mutants marked with * showed non-wild-type phenotypes in other
764 phenotypic screens, as listed in Table S5. Insertions within the EDB cluster are
765 highlighted in bold.

766 [2]Corresponding ORF in *Pseudomonas fluorescens* Pf-5.

767 [3]Orientation of reporter cartridge in transposon relative to disrupted gene (+ =
768 expressed, - = reverse; -/+ = both expressed and reverse orientation insertion
769 mutants isolated).

[4] The sequence flanking insertion 25G4 shows similarity to a region of Pf-5 adjacent to PFL_4308 that is annotated as intergenic and to ORFs annotated as integration host factor in other pseudomonad genomes.

776 **Table 3:** EDB cluster ORFs and their predicted functions

EDB ORF (Pf-5 equivalent)	**Predicted function**	**Score**	**I-Tasser best functional prediction**	**EC score**
1 (Pfl_5540)	SNARE associated Golgi protein (1) 3-dehydroquinate synthase (1)	7.2 e-05 9.2 e-81	Dehydroquinate synthase	0.7656
2 (Pfl_5541)	UbiA prenyltransferase (1)	1.5 e-04	Alginate lyase	0.4716
3 (Pfl_5542)	Sugar phosphate isomerase/epimerase (2)	4 e-06	3',5'-cyclic-nucleotide phosphodiesterase	0.5503
4 (Pfl_5543)	TatD (amino) hydrolase (1)	2.5 e-42	Dihydroorotase	0.9863
5 (Pfl_5544)	Xylose isomerase-like TIM barrel (1)	6.4 e-02	Xylose isomerase	0.5504
6 (Pfl_5545)	Type I phosphodiesterase / nucleotide pyrophosphatase (1)	1.8 e-86	Arylsulfatase	0.6219
7 (Pfl_5546)	No hits to genes with predicted function *	-	Phenol hydroxylase	0.4707
8 (Pfl_5547)	Chromopyrrolic acid synthase, *vioB*	9 e-58	Oligoxyloglucan	0.4823

	(2) *		reducing end-specific cellobiohydrolase	
9 (Pfl_5548)	No hits to genes with predicted function	-	Beta-mannanase	0.5066
10 (Pfl_5549)	No hits to genes with predicted function *	-	Inositol polyphosphate 1-phosphatase	0.6464
11 (Pfl_5550)	Chromopyrrolic acid synthase, *vioB* (2) *	6 e-08	Methanol dehydrogenase	0.3808
12 (Pfl_5551)	No hits to genes with predicted function	-	Non-specific serine/threonine protein kinase	0.5152

Predicted functional assignments are based on (1) Pfam domain with annotation (e value < 0.1) or (2) BLASTp hit to gene with this annotation (e value <0.001). E values are given in brackets. Highest scoring I-TASSER functional predictions are based on top EC score (EC-score >1.1 signifies a prediction with high confidence). *ORF 7/ORF 10 and ORF 8/ ORF 11 yield BLAST hits to each other.

781

Titles and Legends to Figures

Figure 1: *P. fluorescens* NZI7 exhibits stable resistance to nematode grazing. **A.** The ability of *C. elegans* to feed on selected pseudomonads, monitored by measuring the area of the bacterial lawn, with *E. coli* OP50 shown for comparison. The area of each bacterial lawn was calculated relative to its area at t=0. Values are the mean of six replicates; error bars = s.d.m. **B.** Comparison of nematode size when fed on selected pseudomonads. After 48 hours eggs and juvenile nematodes were visible on all but NZI7 and Pf-5 treatments. Statistically significant differences in nematode size on NZI7 compared to strains other than Pf-5 (99% confidence, Bonferroni test applied) by One Factor ANOVA (bacterial treatment) were apparent 24 hours after transfer of nematodes to the plates ($F=97.51$, $p<0.01$, $n=10$). Error bars = s.d.m. *Eco* OP50 = *E. coli* OP50 (black circles); *Pfl* SBW25 = *P. fluorescens* SBW25 (white circles); *Psy* DC3000 = *P. syringae* pv. tomato DC3000 (black diamonds); *Pto* NCPPB 2192 = *P. tolaasii* NCPPB 2192 (white diamonds); *Pfl* NZI7 = *P. fluorescens* NZI7 (black triangles); *Pfl* Pf-5 = *P. fluorescens* Pf-5 (white triangles).

Figure 2: Bioassay for identifying NZI7 mutants that show a reduced ability to repel *C. elegans*. NZI7 transposon mutants were replicated onto NGM plates using a 48-pin replicator. Two colonies of *E. coli* OP50 were inoculated at positions marked X on opposite sides of the mutant grid to serve as a food source while nematodes explored the plate. Plates were incubated for two days to allow bacteria to grow and then 20 L4/adult nematodes were placed adjacent to the X positions using a wire loop. The position of an edible mutant that has lost the ability to repel *C. elegans* is highlighted and enlarged in the inset. The “tracks” of uneaten bacteria left by nematodes moving across the plate show that nematodes come into direct contact with colonies of nematode resistant bacteria. Photograph taken 3 days after nematode transfer.

Figure 3: Transposon insertions in the EDB gene-cluster that disrupt the nematode repellent phenotype of NZI7. Transposon insertions in eight 'edible' mutants of *P. fluorescens* NZI7 mapped to a 20-Kb region of the previously sequenced *P. fluorescens* Pf-5 chromosome. The positions of the insertions are indicated in purple. Predicted genes are indicated in blue (forward strand) and red (reverse strand). Regions of sequence similarity (as detected by BLAST with an E-value threshold of 1e-6) between Pf-5 (RefSeq:NC_004129) and the draft genome assembly of NZI7 are indicated in green. The image was generated using CGView software (Stothard and Wishart 2005).

Figure 4: The EDB gene cluster and the global regulator GacS are required for resistance to nematode predation. A. Complementation of the EDB mutant 79G1 with the EDB cluster restores the ability to repel *C. elegans.* Lawns of NZI7, 79G1, the complemented strain NZI7 79G1 (pLAF4EDB) and the vector control NZI7 79G1 (pLAFR6) inoculated with nematodes and photographed after 3 days. Scale bar = 1 mm. **B.** In choice assays nematodes inoculated at point X consume mutant 79G1 and 79G1 (pLAFR6) but fail to consume wild-type NZI7 and 79G1 (pLAF4EDB). A representative plate photographed three days after addition of ~20 L3/L4 nematodes is shown. **C.** *gacS* mutants support faster nematode growth than NZI7 or 79G1. Nematodes were inoculated onto lawns of NZI7, the EDB mutant 79G1 and the *gacS* mutant 21A6. A statistically significant difference (95% confidence, Bonferroni test applied) in nematode size was observed between nematodes fed *gacS* and wild-type bacteria within 24 hours after inoculation (One Factor ANOVA, $F=152.01$, $p<0.01$, $n=10$). Statistically significant differences in nematode size between all three strains were observed 48 hours after inoculation ($F=770.97$, $p<0.01$, $n=10$). Error bars= s.d.m. **D.** EDB mutants show higher resistance to nematode predation compared to *gacS* mutants. Bacterial lawns with equivalent cell numbers were created by spotting

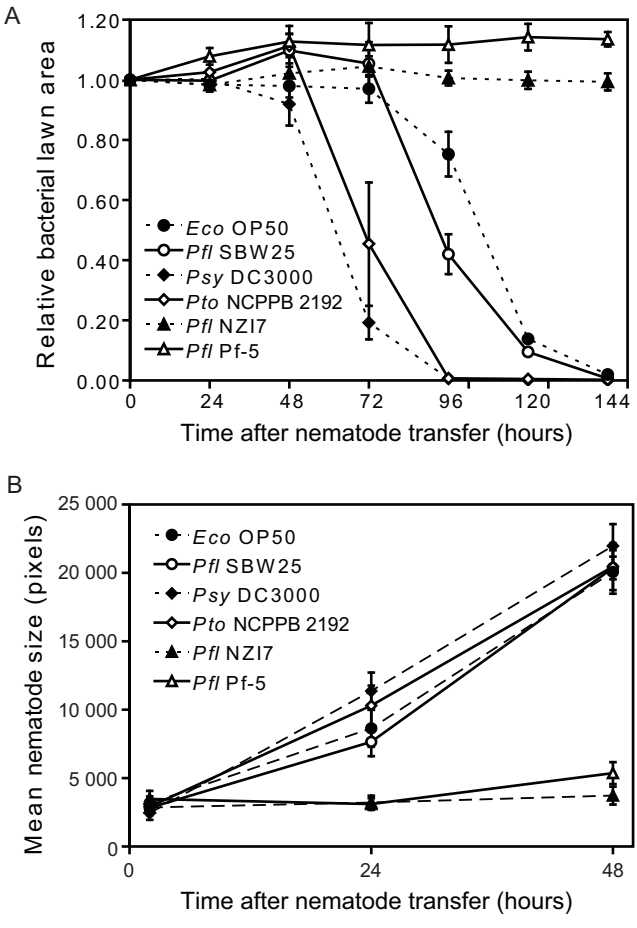
50 µl of an overnight culture adjusted to an OD_{600} of 0.1 at the centre of an NGM plate and incubating plates for 24 hours. Individual lawn areas were normalized relative to day 0 when nematodes were added to the plates. Statistically significant differences (95% confidence, Bonferroni test applied) are indicated with letters (One Factor ANOVA, F= 164.81, P<0.001, n=6). Error bars= s.d.m. EDB = transposon mutant 79G1; *gacS* = transposon mutant 21A6; pLAF4EDB = EDB gene cluster cloned in pLAFR6; pLAFR6 = vector control; pME3258 = *gacS* cloned in pRK415; pRK415 = vector control.

Figure 5: Nematodes show greater attraction to *gacS* mutants than to EDB mutants in chemotaxis assays. Nematode chemotaxis was assessed by inoculating an NGM plate with the two bacterial strains to be tested, incubating the plate for 24 hours, and then inoculating approximately 200 L3/L4 nematodes into the centre of the plate. The number of nematodes at each bacterial colony was counted six hours after transfer. The chemotaxis index was calculated as (number on spot 2 - number on spot 1) / total number of nematodes at both spots. Letters indicate statistical groupings of means for comparisons to the same reference strain (indicated above). Statistically significant differences (95% confidence, Bonferroni test applied) were assessed by One Factor (bacterial treatment) ANOVA (F_{OP50}= 10.01, p<0.01, n=6; F_{NZI7}=8.24, p<0.01, n=6; F_{gacS}=7.97, p<0.01, n=6). Error bars = s.e.m. Pairwise comparisons of OP50:OP50, NZI7:NZI7 and NZI7 *gacS*: NZI7 *gacS* gave chemotaxis indices close to zero.

Figure 6: Expression of the EDB gene cluster is dependent on GacS. A. Photon counting camera image showing that deletion of *gacS* strongly reduces *lux* expression in mutant NZI7 79G1 (EDB ORF1), in which the promoterless *lux*

cartridge in the transposon is oriented in the same direction as the EDB cluster, providing a reporter of EDB expression. Five independent NZI7 79G1 *ΔgacS* mutants are shown, streaked in sectors on an LB agar plate. The sixth sector (marked with an asterisk) contains the parent strain, mutant NZI7 79G1. **B**. Deletion of *gacS* strongly reduces *lux* expression in mutant NZI7 54G10 (EDB ORF8) and in mutant NZI7 5e3 (tolaasin-like toxin (TOL)). Luminescence is plotted as relative light units (RLU) normalised for optical density at 600 nm (indicative of bacterial growth). Strains were cultured in LB medium (supplemented with antibiotics where necessary) over 24 hours; hourly readings are shown. Error bars= s.e.m; n= 6. **C.** The EDB genes constitute an operon that is regulated by GacS. RT-PCR primers targeted to ORF8 of the EDB gene cluster were used to compare mRNA expression in NZI7, *gacS* mutant 21A6, and EDB mutants 79G1 (ORF1) and 54G10 (ORF8). The transposon insertion in 54G10 is located downstream of the RT-PCR primers used. Expression of the housekeeping gene *rpoD* is shown for comparison. **D.** Complementation of the EDB/gacS double mutant NZI7 79G1 *ΔgacS* with *gacS* (pME3528) restores EDB expression. NZI7 79G1 *ΔgacS* (pRK415) is included as a vector control. Strains were cultured in LB medium (supplemented with antibiotics where necessary) over 24 hours; hourly readings are shown. Error bars= s.e.m, n= 10.

Figure 1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1.00
0.80
0.60
0.40
0.20
0.00
0
24
48
72
96
120
144
Time after nematode transfer (hours)
Eco OP50
Pfl SBW25
Psy DC3000
Pto NCPPB 2192
Pfl NZI7
Pfl Pf-5
B
Mean nematode size (pixels)
25 000
20 000
15 000
10 000
5 000
0
0
24
48
Time after nematode transfer (hours)
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Pfl SBW25
Psy DC3000
Pto NCPPB 2192
Pfl NZI7
Pfl Pf-5

Figure 2

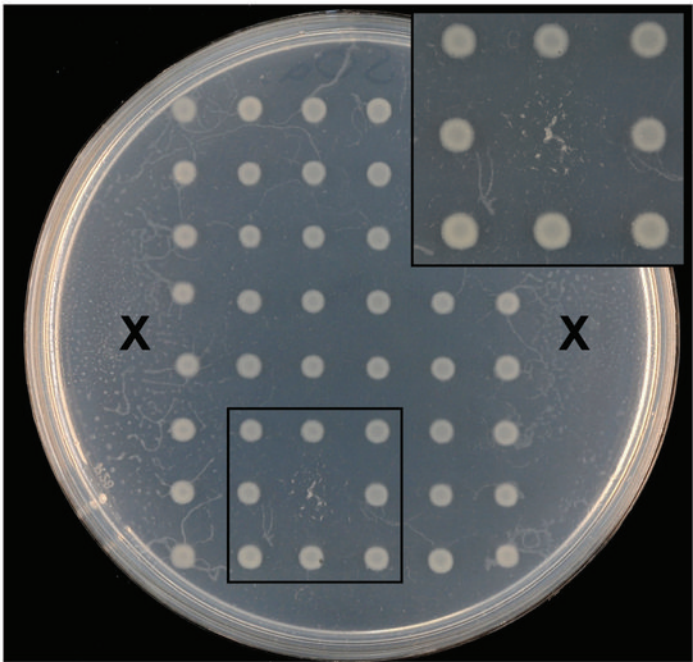
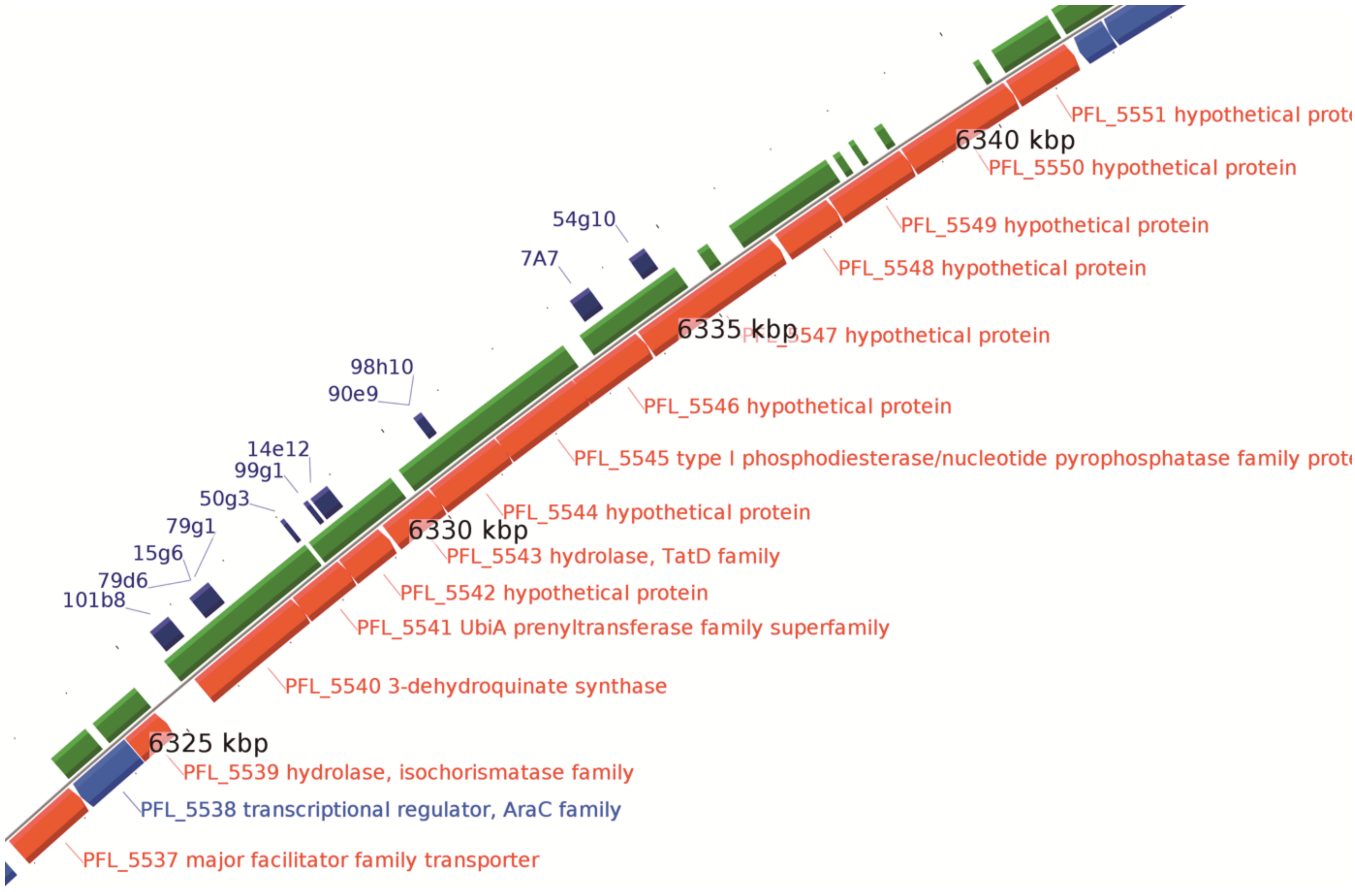


Figure 3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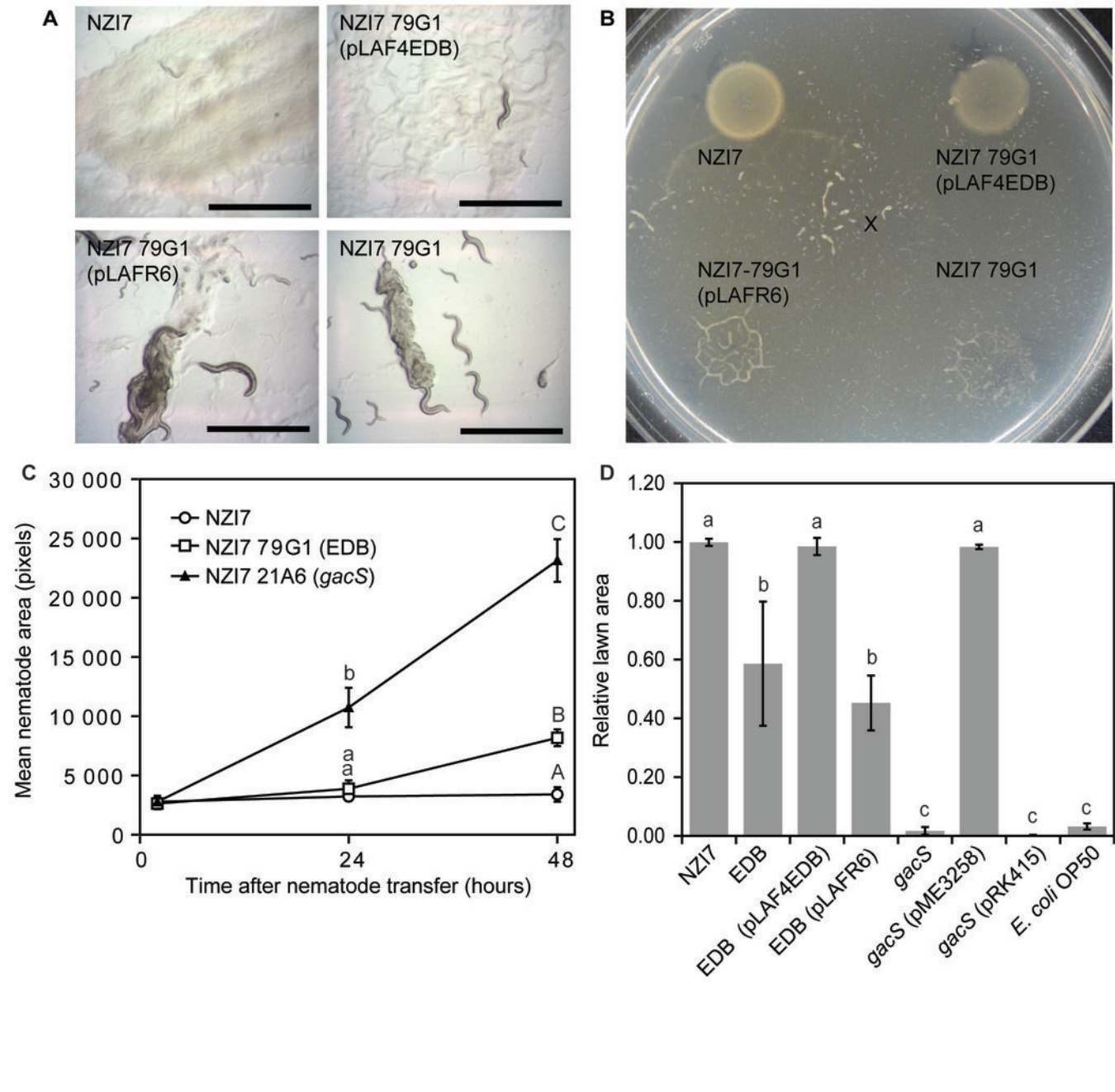
101b8

Figure 4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NZI7
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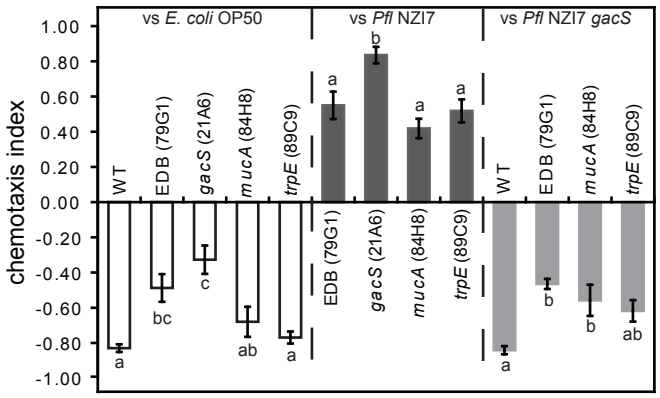
NZI7 79G1
(pLAFR6)
NZI7 79G1
B
NZI7
NZI7 79G1
(pLAF4EDB)
X
NZI7-79G1
(pLAFR6)
NZI7 79G1
C
Mean nematode area (pixels)
30 000
25 000
20 000
15 000
10 000
5 000
0
NZI7
NZI7 79G1 (EDB)
NZI7 21A6 (gacS)
C
b
B
a
a
A
0
24
48
Time after nematode transfer (hours)
D
Relative lawn area
1.20
1.00
0.80
0.60
0.40
0.20
0.00
a
b
a
b
c
a
c
c
NZI7
EDB
EDB (pLAF4EDB)
EDB (pLAFR6)
gacS
gacS (pME3258)
gacS (pRK415)
E. coli OP50

Figure 5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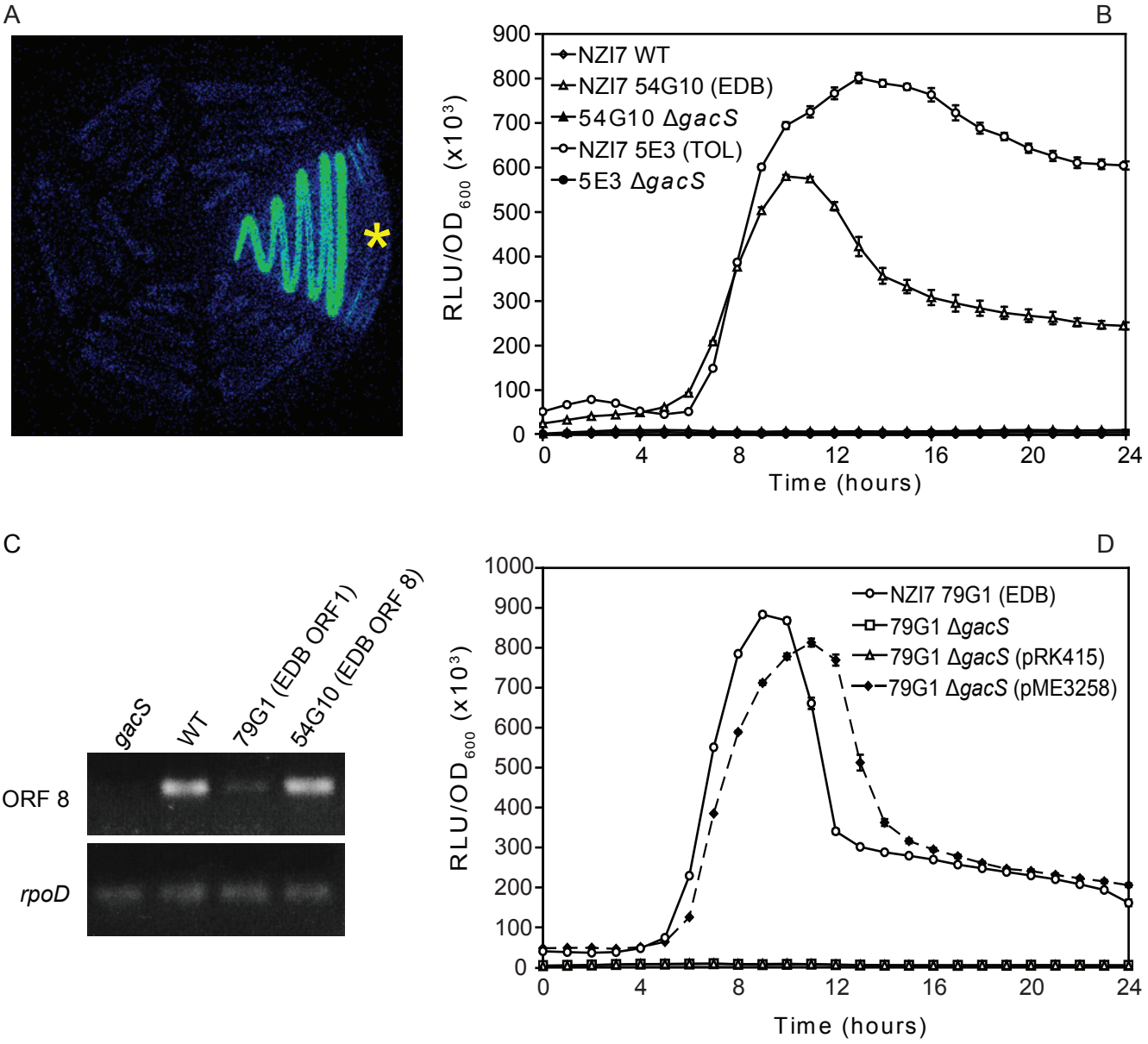
0.80
0.60
0.40
0.20
0.00
-0.20
-0.40
-0.60
-0.80
-1.00
vs E. coli OP50
vs Pfl NZI7
vs Pfl NZI7 gacS
WT
EDB (79G1)
gacS (21A6)
mucA (84H8)
trpE (89C9)
a
bc
c
ab
a
a
b
a
a
a
b
b
b
ab

Figure 6


A
B
C
D
NZI7 WT
NZI7 54G10 (EDB)
54G10 ΔgacS
NZI7 5E3 (TOL)
5E3 ΔgacS
RLU/OD600 (x10^3)
Time (hours)
gacS
WT
79G1 (EDB ORF1)
54G10 (EDB ORF 8)
ORF 8
rpoD
NZI7 79G1 (EDB)
79G1 ΔgacS
79G1 ΔgacS (pRK415)
79G1 ΔgacS (pME3258)