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ORIGINAL ARTICLE

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

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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* is frequently associated with cultivation of the edible mushroom *Agaricus bisporus,* and has been reported to increase the severity of bacterial blotch of mushrooms, a disease caused by bacteria from the *Pseudomonas fluorescens* complex. 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 related to the biocontrol strain *P. protegens* 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'). Our results suggest that the product of the EDB cluster is a poorly diffusible or cell-associated factor that acts together with other features of NZI7 to provide a novel mechanism to deter nematode grazing. As nematodes interact with NZI7 colonies before being repelled, the EDB factor may enable NZI7 to come into contact with and be disseminated by *C. elegans* without being subject to intensive predation.

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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 *Caenorhabditis elegans* (Tan *et al.,* 1999, O'Quinn *et al.,* 2001; Sifri *et al.,* 2005; Powell and Ausubel, 2008; Troemel *et al.,* 2008; Bjornlund *et al.,* 2009; Pedersen *et al.,* 2009; Tampakakis *et al.,* 2009; Zaborin *et al.,* 2009; Irazoqui *et al.,* 2010; Niu *et al.,* 2010; Rae *et al.,* 2010). 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 (Zhang *et al.,* 2005; Shtonda and Avery, 2006; Pradel *et al.,* 2007; Ha *et al.,* 2010). However, studies examining the interaction of *C. elegans* with bacteria it encounters in the environments in which it is commonly found are relatively scarce (Félix and Braendle, 2010; Freyth *et al.,* 2010).

The model strain *C. elegans* strain N2 was originally isolated from a mushroom farm (Hansen *et al.,* 1959; Grewal and Richardson, 1991; Chen *et al.,* 2006). *C. elegans* has also been isolated from compost, snails, rotting fruit and wild decaying mushrooms (Hodgkin and Doniach, 1997; Barriere and Felix, 2005; Caswell-Chen *et al.,* 2005; Barriere and Felix, 2007). On mushroom farms *C. elegans* is known to colonise the fruiting bodies (sporophores)

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of *Agaricus bisporus,* where bacteria belonging to the genus *Pseudomonas* commonly occur (Grewal and Richardson, 1991; Grewal, 1991a). Synergistic interactions between *C. elegans* and mushroom pathogenic pseudomonads have been reported to increase the severity of blotch disease of cultivated mushrooms (Grewal, 1991a,b). 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 a mushroom farm environment, *C. elegans* may be likely to interact more frequently with pseudomonads than with bacteria that are commonly used as food sources in laboratory studies, such as *Escherichia coli* (Zarkower *et al.,* 1984; Atkey *et al.,* 1992; Grewal and Hand, 1992; Tsukamoto *et al.,* 2002; Viji *et al.,* 2003; Curran *et al.,* 2005). 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 *Pseudomonas 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 medium (Sambrook and Russell, 2001) at 28C and 37 °C, respectively, for 24 h before use. Strains used are listed in Supplementary Table S1. Wild-type *C. elegans* Bristol N2 and *Caenorhabditis briggsae* nematodes were maintained at either 15, 20 or 22 °C 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^{-1}, tetracycline 20 μg ml^{-1}, chloramphenicol 25 μg ml^{-1}. Nematode rapid killing assays were performed on brain heart infusion (BHI) agar (Oxoid, Hampshire, UK).

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 h at 28 °C. Ten synchronous L2/L3 nematodes were transferred to each plate and plates were incubated at 20 °C. 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, CA, USA). Mixed bacterial populations were tested similarly, except that overnight cultures were mixed in different ratios before 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 2 days at 28 °C. Nematode preference was assessed by placing ~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 °C. 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 6 h 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 h at 28 °C and then between 15 and 20 L3/L4 nematodes were transferred to the centre of the lawns. Plates were incubated at 20 °C 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 mini-Tn5::*gfp::lux* is described in Supplementary Methods. Oligonucleotides used are listed in Supplementary 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 2 days at 28 °C to allow colonies to grow. After incubation, ~20 L4/adult nematodes were placed adjacent to the *E. coli* colonies. Plates were incubated for 3 days at 20 °C 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 h and then placing ~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,

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

Strain		*Rank*[a]	*Observations (0–72 h)*
P. fl	**NZ047**[b]	1+	Egg laying at <44 h
P. fl	WCS365	1+	Egg laying at <44 h
P. fl	**NZ062**	1+	Egg laying at <44 h
P. fl	**NZ113**	1+	Egg laying at <44 h
P. to	**NCPPB 2192**	1+	Egg laying at <44 h
P. fl	**NZ009**	1+	Egg laying at <44 h, long
P. fl	**NZ43**	1+	Egg laying at <44 h
P. fl	**NZ092**	1	Egg laying within 46 h
P. sy	DC3000	1	Egg laying within 46 h
P. ae	**NZ017**	1	Egg laying within 46 h
P.sy	B728a	1	Egg laying within 46 h
P. sy	1448A	1	Egg laying within 46 h
P. vi	PC006	1	Egg laying within 48 h
P. en	L48	2	Egg laying within 48 h, similar to OP50
P. fl	**NZ065**	2	Egg laying within 48 h
P. sy	PN2	2	Egg laying within 48 h
P. sy	870A	2	Egg laying within 48 h
P. fl	**NZ102**	2	Egg laying within 48 h
P. fl	**NZ043**	2	Egg laying within 48 h
P. fl	**NZ096**[c]	2	Egg laying within 48 h
P. fl	**NZ060**	2	Egg laying within 48 h
P. gi	**NCPPB3146**	2	Egg laying within 48 h
P. fl	WH6	2	Egg laying within 48 h
P. fl	SBW25	2	Egg laying within 48 h
P. fl	**NZ024**	2	Egg laying within 48 h
P. fl	**NZ014**	2	Egg laying within 48 h
P. fl	**NZ104**[c]	2	Egg laying within 48 h
P. fl	EJP115[c]	3	Some growth delay
P. re	**NCPPB 387**	3	Some growth delay, no eggs after 48 h
P. fl	WCS417	3	Variable sizes, few eggs, fertility inhibited
P. fl	OE28.3	3	Many adults after 48 h
P. fl	**NZ112**[c]	3	Some eggs at 48 h, many adults, sluggish
P. sy	B301D	3	Egg laying within 48 h, loopy
P. fl	**NZ006**	3	Egg laying within 50 h
P. fl	**NZ103**[c]	3	Egg laying within 50 h
P. fl	**NZ031**	3	Egg laying within 50 h, thin, uncoordinated
P. fl	**NZ011**[c]	3	Egg laying within 50 h, thin
P. fl	**NZ007**	3	Egg laying within 50 h, thin
P. fl	BBc6R8	3	Some growth delay, lawn well grazed[d]
P. fl	EJP116[c]	4	Some growth delay, small adults
P. fl	**NZ039**[c]	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 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**[c]	7	Growth strongly inhibited, lawn ungrazed
P. ch	PCL1391[c]	7	Growth strongly inhibited, lawn ungrazed
P. fl	**NZ101**[c]	8	Dead within hours, tracks visible
P. pr	Pf-5[c]	8	Dead within hours, few tracks
P. fl	**NZ097**[c]	8	Dead
P. ma	CTA23	8	Dead

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

Table 1 (Continued)

Rank Phenotype
7 Nematodes strongly inhibited, lawn ungrazed, no egg production
8 Nematodes dead within h

Abbreviations: *P. ae*, *Pseudomonas aeruginosa*; *P. en*, *Pseudomonas entomophila*; *P. fl*, *Pseudomonas fluorescens*; *P. gi*, *Pseudomonas gingeri*; *P. pr*, *Pseudomonas protegens*; *P. re*, *Pseudomonas reactans*; *P. sy*, *Pseudomonas syringae*; *P. to*, *Pseudomonas tolaasii*; *P. vi*, *Pseudomonas viridiflava*.
[a]The rank of each strain was assigned according to the key below.
[b]Bold font indicates that a strain was isolated from a mushroom farm.
[c]Indicates that the strain was observed to cause rapid killing on brain heart infusion (BHI) agar.
[d]Grazed/ungrazed refers to the extent to which the bacterial lawn was visibly depleted over time through nematode predation.

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 *C. 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. protegens* Pf-5 (formerly *P. fluorescens* Pf-5 (Ramette *et al.,* 2011), and 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

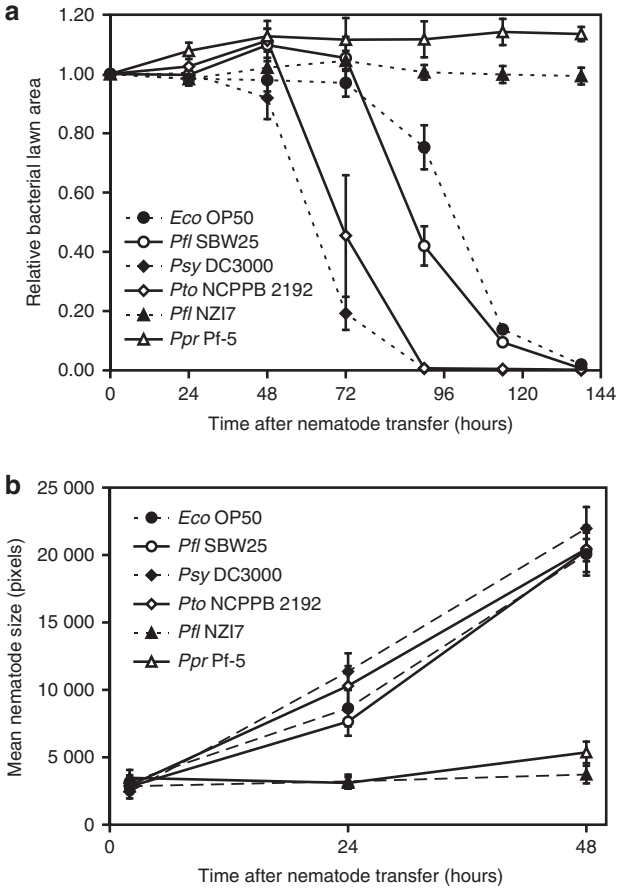

Figure 1 *P. fluorescens* NZI7 exhibits stable resistance to nematode grazing. (**a**) The ability of *C. elegans* to feed on selected pseudomonads, monitored by measuring the area of the bacterial lawn, with *E. coli* OP50 shown for comparison. The area of each bacterial lawn was calculated relative 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 h eggs and juvenile nematodes were visible on all but NZI7 and Pf-5 treatments. Statistically significant differences in nematode size on NZI7 compared with strains other than Pf-5 (99% confidence, Bonferroni test applied) by one factor ANOVA (bacterial treatment) were apparent 24 h 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); *Ppr* Pf-5 = *P. protegens* Pf-5 (white triangles).

Chang *et al*. (2011) has demonstrated that pathogen avoidance by *C. elegans* can be affected by the lateral outer labial mechanosensory head neurons. *tax* mutants feeding on NZI7 grew well and showed no evidence of deleterious effects or pharyngeal dysfunction, allowing us to conclude that NZI7 is not intrinsically pathogenic to *C. elegans* and that the failure of wild-type nematodes to feed on it was due only to aversion and did not involve additional physical effects such as the pharyngeal blockage observed for *C. elegans* feeding on *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. protegens Pf-5

NZI7 causes brown blotch disease on mushrooms, which was attributed to production of a lipodepsipeptide similar to tolaasin (Godfrey *et al.,* 2001a). However, although NZI7 produces a diffusible molecule that behaves like tolaasin in the white line bioassay it is more closely related to Pf-5 than to the tolaasin-producing type strain *P. tolaasii* NCPPB 2192 (Supplementary 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 ($\sim 8\times$ 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 (Supplementary 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 (Supplementary Table S3) including genes 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 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; Siddiqui *et al.,* 2005; Meyer *et al.,* 2009; Neidig *et al.,* 2011). However, one recent study has shown that purified 2,4-diacetylphloroglucinol 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 (Supplementary Table S4) are not obviously associated with nematode repellence or toxicity. 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 (for example, 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*::*lux* 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 of wild-type NZI7 colonies (data not shown). The library was also used to identify mutants with alterations in other traits that might affect mushroom pathogenesis or nematode predation: lipase, chitinase and lecithinase activity, cyanide production, haemolysis and production of the tolaasin-like toxin (TOL) (Supplementary Table S5). Transposon insertion points were determined by two step semi-degenerate PCR (Jacobs *et al.,* 2003).

A total of 84 NZI7 transposon mutants (0.87% of the total, summarised in Table 2) were identified and validated as 'edible'. Of these 84 mutants, 45 displayed a wild-type phenotype in screens for cyanide, exoenzyme and toxin production (Supplementary Table S5). Sequencing led to the determination of insertion points for 75 mutants. Overlay of transposon hits onto corresponding ORF positions in the draft NZI7 genome, mapped onto the complete genome of Pf-5, showed that none of these mutants were unique to NZI7 (Supplementary Figure S2). However, 11 'edible' mutants that had wild-type phenotypes in the other screens performed were associated with a single gene cluster of unknown function, present in both NZI7 and Pf-5 (Figure 3), which is henceforth referred to as the EDB (edible) gene cluster. The region of the NZI7 genome containing the complete EDB cluster (ORFs 1–12) and flanking ORFs (equivalent to PFL_5539 to PFL_5553 in Pf-5) was sub-cloned into the cosmid pLAFR6 (Huynh *et al.,* 1989) to create pLAF4EDB and transformed into NZI7 EDB mutants. The presence of pLAF4EDB restored the ability of EDB cluster mutants to repel *C. elegans*, confirming the role of the EDB cluster in nematode repellence (Figure 4).

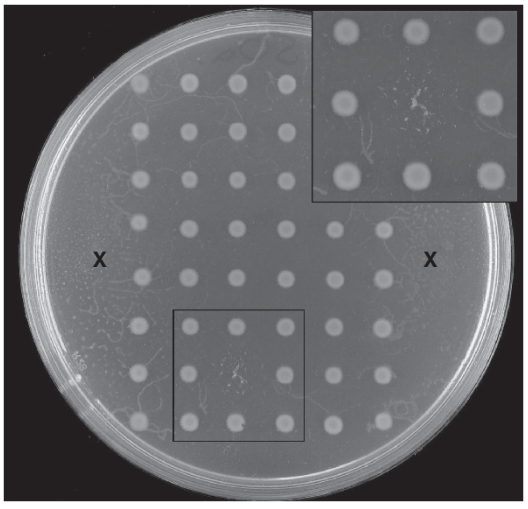

Figure 2 Bioassay for identifying NZI7 mutants that show a reduced ability to repel *C. elegans*. NZI7 transposon mutants were replicated onto NGM plates using a 48-pin replicator. Two colonies of *E. coli* OP50 were inoculated at positions marked X on opposite sides of the mutant grid to serve as a food source while nematodes explored the plate. Plates were incubated for 2 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.

Several of the 'edible' mutants identified in the screen were found have insertions in genes previously shown to be important in *P. aeruginosa* pathogenesis towards *C. elegans*: the global virulence regulator *gacS*; *dsbA,* required for correct folding of periplasmic disulphide-bonded proteins; the phosphoenolpyruvate-protein phosphotransferase gene *ptsP*; purine biosynthesis genes; the two-component sensor histidine kinase *cbrA,* and a haemagglutinin repeat protein with weak similarity to PA0041 (Tan *et al.,* 1999; Gallagher and Manoil, 2001; Tan, 2002; Yorgey *et al.,* 2001). An edible mutant containing an insertion close to the 3′ end of *algU*, and immediately upstream of the *algU* regulatory proteins *mucA* and *mucB,* was observed to have a mucoid phenotype, which has been reported to result in loss of pathogen avoidance in *P. aeruginosa* interactions with *C. elegans* (Reddy *et al.,* 2011). Edible mutants with insertions in genes with predicted roles in aromatic amino acid metabolism (anthranilate and chorismate synthase genes) and thiamine biosynthesis could be chemically complemented by addition of 1 mM anthranilate or thiamine to the growth medium, respectively (Supplementary Figure S3).

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

Delineation and bioinformatic analysis of the EDB gene cluster

The EDB gene cluster is highly conserved in both NZI7 and Pf-5 (Figure 3, Supplementary Table S6). EDB-like gene clusters were also detected in the genomes of several other pseudomonads: *P. fluorescens* WH6 (Kimbrel *et al.,* 2010), *Pseudomonas brassicacearum* NFM421 (Ortet *et al.,* 2011), *P. brassicacearum* Q8r1-96 (Loper *et al.,* 2012), and the insect pathogen *Pseudomonas entomophila* L48 (Vodovar *et al.,* 2006b). We have also identified

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

Mutant[a]	*Identity*	*Best hit*[b]	*Rep*[c]
7F10*	Thiol:disulphide 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*	RNA polymerase sigma factor AlgU[d]	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	Haemagglutinin 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[e]	+
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*	Two-component sensor histidine kinase CbrA	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-SNARE domain protein**[f]	**PFL_5540**	−/+
50G3	**UbiA prenyltransferase family protein**	**PFL_5541**	−
14E12, 99G1	**Hypothetical protein (Sugar phosphate isomerase/epimerase**[g]**)**	**PFL_5542**	+
90E9, 98H10	**Hypothetical protein (Xylose isomerase-like TIM barrel**[f]**)**	**PFL_5544**	+
7A7	**Hypothetical protein (Type I phosphodiesterase**[f]**)**	**PFL_5545**	−
54G10	**Hypothetical protein (Chromopyrrolic acid synthase, *vioB*[g])**	**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	−

[a]Mutants or sets of mutants marked with * showed non-wild-type phenotypes in other phenotypic screens, as listed in Supplementary Table S5. Insertions within the EDB cluster are highlighted in bold.
[b]Corresponding ORF in *P. protegens* Pf-5.
[c]Orientation of reporter cartridge in transposon relative to disrupted gene (+ = expressed, − = reverse; −/+ = both expressed and reverse orientation insertion mutants isolated).
[d]This insertion lies close to the end of *algU*, which is upstream of and in a potential operon with the *algU* regulatory proteins *mucA* and *mucB*.
[e]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.
[f]Pfam domain prediction.
[g]BLASTp hit (*e*-value <0.001).

EDB-like gene clusters in the draft genomes of other mushroom pathogenic pseudomonads, including *P. fluorescens* NZ007, *P. tolaasii* NCPPB2192, *P. tolaasii* PMS117S and *P. gingeri* NCPPB3146 (Supplementary Figure S5). The genomic context of the EDB cluster is not conserved in all of these strains, but four additional ORFs found downstream of the NZI7 EDB cluster genes, corresponding to Pfl_5548-Pfl_5551 in Pf-5, are adjacent to the EDB-like genes in all but two of these strains (Supplementary 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

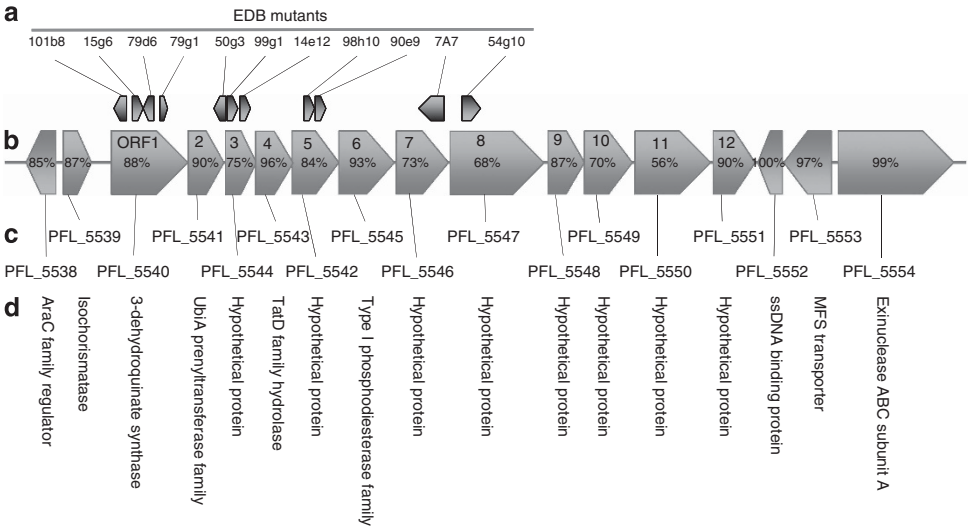

Figure 3 Transposon insertions in the EDB gene cluster that disrupt the nematode repellent phenotype of NZI7. (**a**) Transposon insertions in 11 'edible' mutants of *P. fluorescens* NZI7 mapped to a previously uncharacterised gene cluster that is largely conserved in NZI7 (GenBank: AJXF00000000.1) and in the previously sequenced *P. protegens* Pf-5 chromosome (RefSeq NZ_004129). The positions of the transposon insertions are indicated in black, with the arrow indicating the orientation of the promoterless *gfp::lux* reporter construct within the transposon. (**b**) Predicted ORFs in the EDB region of NZI7. The top row of numbers indicates the ORF numbers referred to in the text, and the second row of numbers indicates percentage identity to the corresponding ORF in *P. protegens* Pf-5. (**c**) Locus tags for corresponding ORFs in *P. protegens* Pf-5. (**d**) Predicted functions of EDB region ORFs.

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, 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 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, Supplementary Table S6). However, the presence of a 3-dehydroquinate synthase domain in the first ORF, a UbiA 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 (Supplementary Table S6).

Bioinformatic analyses using PSORTdb V2.0 (http://www.psort.org/psortb/ (Rey *et al.*, 2005)) and Phobius (http://phobius.sbc.su.se/ (Käll *et al.*, 2004)) indicated that the putative 3-dehydroquinate synthase-like and UbiA prenyltransferase-like 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 3-dehydroquinate synthase proteins as 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) synthesised 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.

GacS regulates expression of the EDB gene cluster

gacS mutant lawns showed the greatest reduction in their ability to repel *C. elegans* (Figures 4 and 5). GacS positively regulates the expression of numerous secreted factors (Rahme *et al.,* 1995; Blumer *et al.,* 1999; Vodovar *et al.,* 2006a; Lapouge *et al.,* 2008) and as expected, *gacS* mutants lacked the ability to produce cyanide, exoenzymes and TOL. However, transposon mutants of NZI7 that lacked the ability to produce these individual traits, and a deletion mutant lacking the 2,4-diacetylphloroglucinol biosynthetic cluster, retained the ability to repel nematodes (Supplementary Table S5, data not shown). This suggested that GacS regulates expression of the EDB cluster along with other unidentified factors that affect NZI7–nematode interactions.

The promoterless *lux* reporter cassette in the mini-Tn5 transposon provides a tool for examining gene expression when the transposon is oriented to place

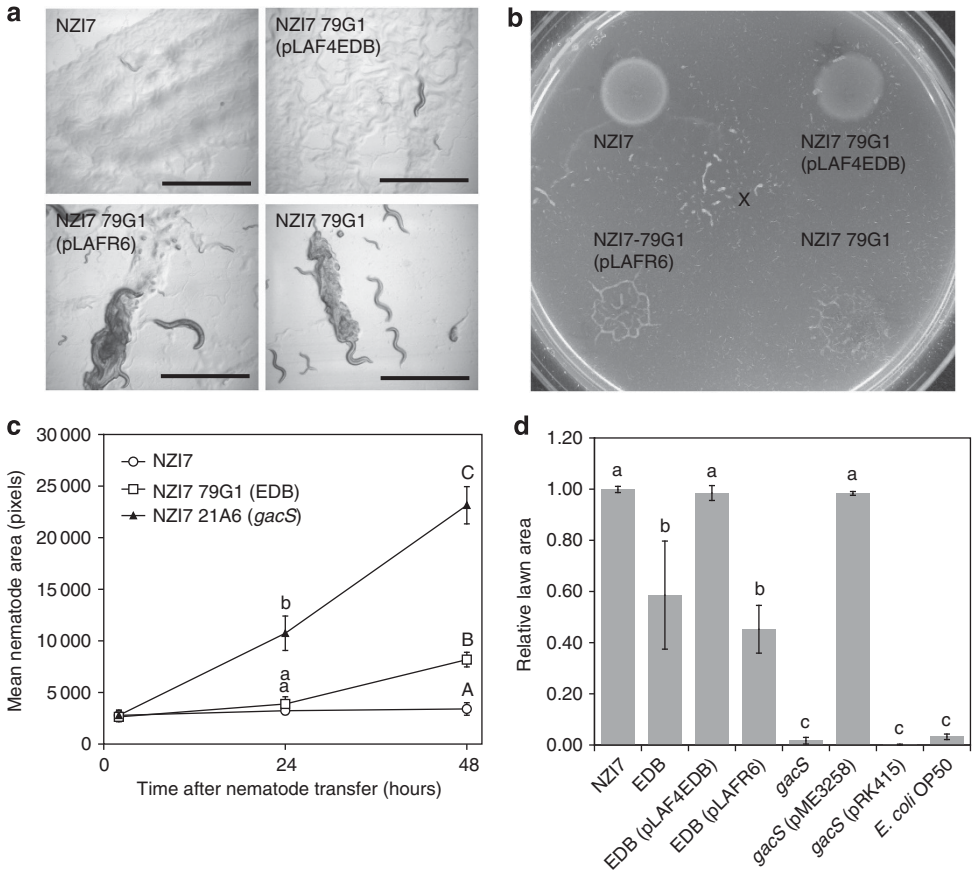

Figure 4 The EDB gene cluster and the global regulator GacS are required for resistance to nematode predation. (**a**) Complementation of the EDB mutant 79G1 with the EDB cluster restores the ability to repel *C. elegans*. Lawns of NZI7, 79G1, the complemented strain NZI7 79G1 (pLAF4EDB) and the vector 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 3 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 h 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 h 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 with *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 h. Individual lawn areas were normalised 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* (Zuber *et al.,* 2003); pRK415 = vector control (Keen *et al.,* 1988).

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 the TOL mutant 5E3 (Figure 6b, Supplementary Table S5). Luminescence in *ΔgacS*/EDB reporter mutants could be restored to wild-type levels by complementation with *gacS* (Figure 6d).

RT-PCR experiments indicated that EDB cluster mRNA was absent in NZI7 *gacS* mutants (Figure 6c) 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 (Whistler *et al.,* 1998; Heeb *et al.,* 2005; Kay *et al.,* 2005). 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

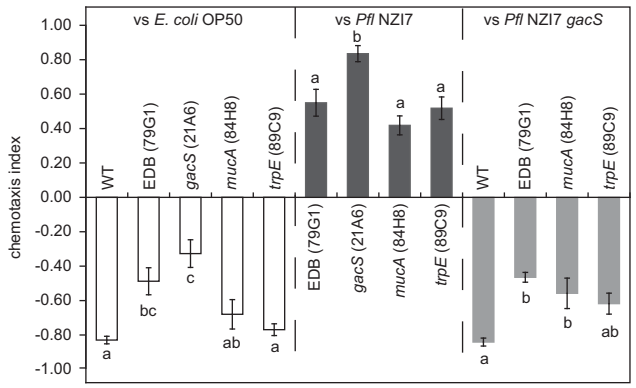

Figure 5 Nematodes show greater attraction to *gacS* mutants than to EDB mutants in chemotaxis assays. Nematode chemotaxis was assessed by inoculating an NGM plate with the two bacterial strains to be tested, incubating the plate for 24 h, and then inoculating ~200 L3/L4 nematodes into the centre of the plate. The number of nematodes at each bacterial colony was counted 6 h 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.

conditions on EDB gene expression. EDB expression was enhanced by mildly acidic pH, rich media, moderate-high iron availability and an optimal carbon-nitrogen balance (Supplementary 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 (Supplementary Figure S6E). Expression was not significantly altered by introduction of the complementing clone pLAF4EDB into reporter strains, indicating that it is not autoregulatory (Supplementary Figure S6F). As EDB mutants continue to produce TOL, cyanide and exoenzymes (Supplementary 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 >5 mm, as the phenotype of EDB mutants would be masked by surrounding colonies if this were the case. *gacS* mutants of *P. protegens* 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). We observed that nematodes fed mixtures of NZI7 wild-type and EDB cluster mutants in different ratios grown on NGM 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 to confirm EDB expression in most of the transformed strains by RT-PCR (Supplementary 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 (Supplementary 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 the region of the NZI7 genome cloned into pLAF4EDB is insufficient to confer resistance to nematode predation. This suggests that NZI7 possesses additional genes that act together with the genes in this region to confer nematode resistance.

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. protegens* 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 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 3 days (Supplementary Figure S8). 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, as 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

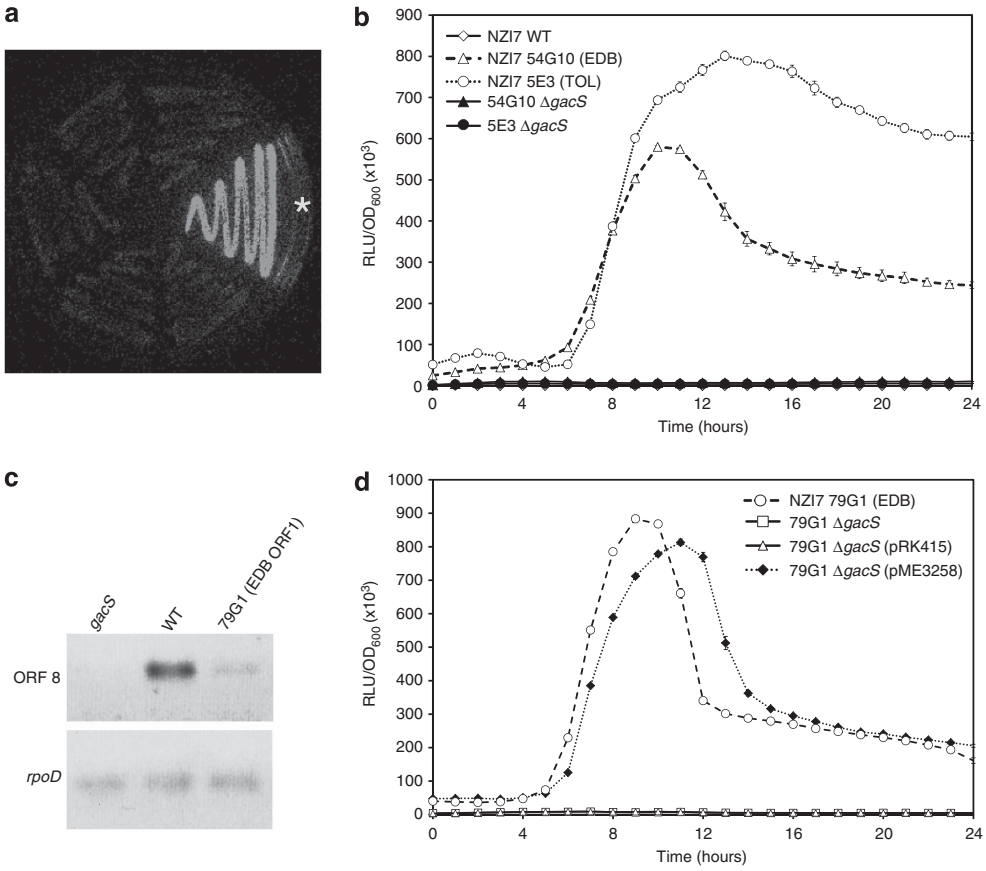

Figure 6 Expression of the EDB gene cluster is dependent on GacS. (**a**) Photon counting camera image showing that deletion of *gacS* strongly reduces *lux* expression in mutant NZI7 79G1 (EDB ORF1), in which the promoterless *lux* cartridge in the transposon is oriented in the same direction as the EDB cluster, providing a reporter of EDB expression. Five independent NZI7 79G1 *ΔgacS* mutants are shown, streaked in sectors on an Luria–Bertani (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 (TOL). Luminescence is plotted as relative light units normalised for optical density at 600 nm (indicative of bacterial growth). Strains were cultured in LB medium (supplemented with antibiotics where necessary) over 24 h; 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 mutant 79G1 (ORF1). Expression of the housekeeping gene *rpoD* is shown for comparison. (**d**) Complementation of the EDB/gacS double mutant NZI7 79G1 *ΔgacS* with *gacS* (pME3258) 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 h; hourly readings are shown. Error bars = s.e.m, $n = 10$.

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. In our assays Pf-5 killed *C. elegans,* which may have masked any repellent activity present, so we do not yet know whether these loci are unique to NZI7 or also present in Pf-5.

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. Although the majority of these mutations require further validation through complementation, it seems likely that many of the disrupted genes also contribute to the nematode repellent phenotype of this strain. Some may be involved in the synthesis of precursor molecules needed for the synthesis of the EDB product,

while others could be involved in the synthesis of a second nematode repellent factor. We also identified an additional class of mutants, exemplified by a mutation affecting the *algU/mucA* operon, where the mucoid phenotype of mutant bacteria may compromise the ability of nematodes to respond to bacterial signals and of bacteria to repel nematode predation (Reddy *et al.,* 2011). It should be noted that mutations affecting the production of additional nematode repellent factors may not have been detected in the grid screen if these factors are highly diffusible or individually contribute only a small fraction of the total NZI7 repellent activity.

C. elegans has been shown to respond to a wide range of chemoattractants and repellents (Ward, 1973; Dusenbery, 1975; Hilliard *et al.,* 2004; Bargmann, 2006) and there is emerging evidence for the perception of bacterial metabolites by nematodes, notably quorum sensing molecules and serrawettin W2 (Beale *et al.,* 2006; Pradel *et al.,* 2007). Thus, the EDB locus may be involved in the synthesis of a molecule that identifies NZI7 as a strain to be avoided, even though the product of the EDB cluster itself is not directly toxic to nematodes. The ability to preferentially feed on non-deleterious bacteria would clearly be advantageous to nematodes in the natural environment, where bacterial populations are heterogeneous (Rodger *et al.,* 2004; Zhang *et al.,* 2005; Laws *et al.,* 2006; Shtonda and Avery, 2006).

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

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