

Pseudomonas brassicacearum Strain DF41 Kills *Caenorhabditis elegans* through Biofilm-Dependent and Biofilm-Independent Mechanisms

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ABSTRACT

Pseudomonas brassicacearum DF41 is a biocontrol agent that suppresses disease caused by the fungal pathogen *Sclerotinia sclerotiorum*. A number of exometabolites are produced by DF41, including the lipopeptide sclerosin, hydrogen cyanide (HCN), and degradative enzymes. The production of these compounds is controlled at both the transcriptional and posttranscriptional levels by quorum sensing (QS) and the Gac two-component regulatory system. In order to be successful, a biocontrol agent must persist in the environment at levels sufficient for pathogen control. Bacterivorous predators, including nematodes, represent a challenge to the establishment of introduced microorganisms. In the current study, DF41 was investigated for its ability to resist predation by *Caenorhabditis elegans*. We discovered that this bacterium is capable of killing *C. elegans* through two different mechanisms: the first involves exposure to toxic metabolites, and the second entails biofilm formation on the nematode head blocking the buccal cavity. Biofilm formation on nematodes, which has been reported only for *Yersinia* spp. and *Xenorhabdus nematophila*, is dependent upon the Gac system. Biofilms were not observed when bacteria were grown on NaCl-containing medium or on *C. elegans* biofilm-resistant mutants. Coculturing with nematodes led to the increased expression of the *pdfRI-rfiA* QS genes and *hcnA*, which is under QS control. HCN was the most nematocidal of the exometabolites, suggesting that this bacterium can respond to predator cues and upregulate expression of toxins accordingly. In summary, DF41 is able to respond to the presence of *C. elegans*, and through two distinct mechanisms, it can escape predation.

IMPORTANCE

Pseudomonas brassicacearum DF41 can suppress fungal pathogens through a process known as biocontrol. To be successful, a biocontrol agent must be able to persist in the environment at levels sufficient for pathogen control. Predators, including the nematode *Caenorhabditis elegans*, represent a threat to persistence. The aim of the current study was to investigate the DF41-*C. elegans* interaction. We discovered that DF41 is able to escape predation through two distinct mechanisms. The first involves exposure to toxic bacterial metabolites, and the second entails the formation of a sticky coating on the nematode head, called a biofilm, which blocks feeding and causes starvation. We report here a pseudomonad forming biofilms on the *C. elegans* surface. When grown with *C. elegans*, DF41 exhibits altered gene expression and metabolite production, indicating that this bacterium can sense the presence of these predators and adjust its physiology accordingly.

The production of extracellular metabolites by biocontrol bacteria is energetically costly; as such, these compounds are expected to impart a fitness advantage to the producer. It can be argued that reduced competition for resources is not sufficient to warrant the synthesis of inhibitory compounds. Rather, these products must provide additional advantages, such as reducing the threat of grazing predators through their repellent and/or cidal activities (1, 2). Nematodes are among the most abundant animals on the planet, and through their grazing pressure, they are believed to influence microbial community structure (3). Bacteria, in turn, have evolved defensive mechanisms to resist nematode grazing, including the production of exometabolites that act to deter and/or reduce predator populations. The model organism *Caenorhabditis elegans* has been widely employed in bacterium-nematode interaction studies because of its genetic tractability and the comprehensive array of mutants available (4). For pseudomonads that exhibit pathogenicity toward *C. elegans*, killing ensues through two distinct mechanisms. On rich media, which support the production of high levels of toxic metabolites, death occurs through intoxication, a mechanism known as fast killing

(5–8). Conversely, low-nutrient media result in the production of sublethal levels of exometabolites, and slow killing occurs over the course of several days. In this instance, bacterial colonization of the intestinal tract ultimately leads to nematode death (8).

In nature, the bulk of bacterial biomass exists as an adherent community of cells encased in an extrapolymeric matrix, collectively known as a biofilm. The ability to form biofilms has many advantages, including protection from environmental assaults that would threaten planktonic cells (9). Biofilms exhibit elevated

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resistance to biocidal agents, immune components, desiccation, and UV radiation (9, 10). For a select group of bacteria, the ability to form biofilms allows them to escape predation by grazing nematodes. In 2002, Darby and coworkers reported that *Yersinia pestis* is able to form biofilms on the head and body of *C. elegans*, blocking feeding and ultimately causing starvation (11). Similarly, *Xenorhabdus nematophila* (12) and some but not all strains of *Yersinia pseudotuberculosis* (13) are capable of establishing nematode-associated biofilms. Screening of an array of 26 pathogenic bacteria revealed that *C. elegans* surface colonization is not a common trait (14, 15). Even the notorious infection-related biofilm formers *Pseudomonas aeruginosa* and uropathogenic *Escherichia coli* were found to lack this ability, leading to the conclusion that this defense strategy is employed by only a few bacterial species (15).

Pseudomonas brassicacearum strain DF41 is a biocontrol agent capable of suppressing disease caused by the plant-pathogenic fungus *Sclerotinia sclerotiorum* (Lib.) de Bary in both greenhouse and field studies (16, 17). This bacterium produces an arsenal of extracellular metabolites, including degradative enzymes, hydrogen cyanide (HCN), and a novel lipopeptide called sclerosin (17, 18). Sclerosin has been confirmed to be essential for biocontrol, as a sclerosin-deficient mutant, DF41-1278, exhibits dramatically reduced fungal suppression (17). A complex regulatory cascade oversees the production of DF41 exometabolites, including the GacS/GacA two-component system, the stationary-phase sigma factor RpoS, and the stringent response (17, 19). In addition, DF41 has a quorum-sensing (QS) system composed of a LuxR-type transcriptional activator (PdfR) and an acyl-homoserine lactone (AHL) synthase (PdfI), encoded by *pdfR* and *pdfI*, respectively (20). Immediately downstream of *pdfI* lies a third gene in the QS locus, called *rflA*. RflA belongs to a distinct group of LuxR regulators characterized as having a C-terminal helix-turn-helix DNA binding motif but lacking an N-terminal AHL binding domain (20, 21). AHL signaling molecules are not involved in DF41 biocontrol, because an AHL-deficient strain exhibits no discernible phenotype (20). RflA, on the other hand, was found to be essential, as an *rflA* mutation results in a complete loss of antifungal activity (20). RflA is believed to control expression of the PdfABC efflux pump involved in exometabolite export. In the $\Delta rflA$ mutant background, metabolites that are normally excreted accumulate to elevated levels within the mutant cells (20).

The aim of the current study was to elucidate the interaction between DF41 and the bacterivorous predator *C. elegans*. Specifically, we were interested to learn whether DF41 is able to resist nematode grazing and what role, if any, DF41 exoproducts play in the bacterium-nematode interaction. We discovered that DF41 is capable of killing *C. elegans* through two distinct modes; the first involves exposure to toxic metabolites, while the second entails biofilm formation on the nematode surface. It has been reported that bacteria are capable of responding to predator cues through altered expression of toxin-encoding genes (7, 22, 23). To see if the same holds true for DF41, we cocultured bacteria with *C. elegans* and monitored the expression of genes associated with exometabolite production. In the presence of *C. elegans*, several genes were upregulated, including *hcnA* (hydrogen cyanide) and the QS genes *pdfRI* and *rflA*. Our findings indicate that through soluble chemical cues and/or direct contact, DF41 is able to perceive the presence of *C. elegans* and adjust its physiology accordingly.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* was cultured at 37°C on lysogeny broth (LB) agar (Difco Laboratories, Detroit, MI). *Pseudomonas* strains were routinely cultured on LB, King's B (KB) (24), or in M9 minimal salts medium supplemented with 0.2% glucose (M9-Glc) at 28°C, unless otherwise indicated. The antibiotics used were 100 µg/ml ampicillin (Amp), 15 µg/ml gentamicin (Gm), and 15 µg/ml tetracycline (Tc) for *E. coli*, and 20 µg/ml Gm, 40 µg/ml piperacillin (Pip), and 15 µg/ml Tc for DF41. All antibiotics were obtained from Research Products International Corp. (Mt. Prospect, IL).

Nematode strains and culture conditions. The *C. elegans* strains used in this work include wild-type Bristol N2, AT6 [*srf-2(yj262)*], DC9 [*bah-3(b9)*], and CB4856, which were maintained at 15°C on nematode growth medium (NGM) (25) supplemented with *E. coli* OP50. The protocols available in WormBook were used in order to obtain the synchronous cultures (26). L4-stage hermaphrodites were used in the studies described herein.

Nucleic acid manipulation. Cloning, purification, electrophoresis, and other manipulations of DNA were performed using standard techniques (27).

Construction of a DF41 *hcn* mutant. To generate an *hcn* mutant, an internal region of the DF41 *hcn* gene cluster was PCR amplified using primers *hcnA-FOR1* and *hcnC-REV1* (Table 1). A TOPO kit (Thermo Fisher Scientific, Waltham, MA, USA) was employed to clone the 1.6-kb PCR product into the pCR2.1-TOPO vector, creating pCR*hcnABC*-41. To liberate the insert, pCR*hcnABC*-41 was digested with HindIII and XhoI and subcloned into the same sites of the suicide vector pKNOCK-Tc. Triparental mating between the donor [*E. coli* DH5α λpir(pKNOCK*hcnABC*)], helper [*E. coli* DH5α(pRK600)], and recipient (DF41) strains was performed to interrupt the wild-type *hcnABC* gene cluster as a single crossover insertion selected using Tc. PCR was used to confirm the *hcn* mutation, and the mutant was found to be devoid of HCN production when tested using Cyantesmo paper (Macherey-Nagel GmbH and Co., Germany).

Nematode slow- and fast-killing assays. Slow-killing assays were performed by spotting 10 µl of a 1:10 dilution of an overnight bacterial culture grown in KB, LB, or NGM onto a 35 by 10-mm agar plate of the same medium. After 24 h of incubation at 28°C, the plates were cooled to room temperature and seeded with 30 L4-stage nematodes. The plates were incubated at 20°C, and the nematodes were scored for viability by examining them with a stereomicroscope over a 10-day period. Nematodes without detectable movement were considered dead after confirmatory prodding with a nematode pick. Three replicates were included for each trial, and the assays were repeated three times independently. Fast-killing assays were carried out in a similar manner, except that brain heart infusion (BHI) agar (Difco) with and without FeCl₃ (100 µM) was used as the medium, and nematodes were monitored at 4-, 6-, 8-, 10-, 12-, and 24-h time points.

Microscopic imaging of *Caenorhabditis elegans*. Bacterial strains harboring pMCh-23, which encodes constitutively expressed mCherry fluorescent protein, were spotted onto agar plates and incubated overnight at 28°C. Plates were cooled to room temperature prior to seeding with nematodes, followed by incubation at 20°C. Nematodes were mounted onto 2% agarose pads on glass microscope slides and anesthetized with 10 mmol/liter levamisole (Sigma) in M9 medium. The nematodes were examined with a Zeiss LSM 700 confocal laser scanning microscope (Carl Zeiss Microscopy GmbH, Göttingen, Germany).

Biofilm formation. To analyze the ability of DF41 and derivative strains to form biofilms, a 96-well plate assay was employed, as detailed in a study by Berry et al. (17).

Analysis of metabolite and regulatory gene expression in DF41 in the presence and absence of *Caenorhabditis elegans*. Gene expression was monitored in DF41 grown in the presence and absence of *C. elegans*. Approximately 200 nematodes were added to a 3-ml volume of DF41

TABLE 1 Bacterial strains, plasmids, and primers used in the study

Strain, plasmid, or primer	Relevant genotype, phenotype, or sequence ^a	Reference or source
Strains		
<i>C. elegans</i>		
N2	Wild-type isolate	CGC, University of Minnesota, MN
AT6	<i>srf-2(yj262)</i>	CGC, University of Minnesota, MN
DC9	<i>bah-3(br9)</i>	CGC, University of Minnesota, MN
CB4856	Wild-type Hawaiian isolate	CGC, University of Minnesota, MN
<i>P. brassicacearum</i>		
DF41	Rif ^r wild type (canola root tip isolate)	16
DF41-1278	Rif ^r <i>lp::Tn5-1063</i> genomic insertion	17
DF41 <i>hcn</i>	DF41 with the pKNOCK-Tc vector inserted into the <i>hcn</i> gene	This study
AI-deficient mutant	DF41 carrying pME6863	20
DF41 <i>rfa</i>	DF41 with Gm ^r cassette inserted into <i>rfa</i> gene	20
DF41 <i>gacS</i>	Rif ^r <i>gacS::Tn5-1063</i> genomic insertion	17
DF41- <i>rfp</i>	DF41 containing mCherry expressed from pMCh-23	18
DF41-1278- <i>rfp</i>	DF41-1278 containing mCherry expressed from pMCh-23	18
DF41 <i>hcn</i> - <i>rfp</i>	DF41 <i>hcn</i> containing mCherry expressed from pMCh-23	This study
AI-deficient- <i>rfp</i> mutant	DF41-6863 containing mCherry expressed from pMCh-23	This study
DF41 <i>rfa</i> - <i>rfp</i>	DF41 <i>rfa</i> containing mCherry expressed from pMCh-23	This study
DF41 <i>gacS</i> - <i>rfp</i>	PA23 <i>gacS</i> containing mCherry expressed from pMCh-23	This study
<i>E. coli</i>		
DH5α	<i>supE44 ΔU169 (Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Gibco
DH5α <i>λpir</i>	<i>λpir</i> lysogen of DH5α	46
OP50	Laboratory strain for maintenance of <i>C. elegans</i> ; uracil auxotroph	25
<i>Chromobacterium violaceum</i> CVO26	Autoinducer synthase (<i>cviI</i>) mutant from <i>C. violaceum</i> ATCC 31532 autoinducer biosensor	29
Plasmids		
pME6863	pME6000 carrying the <i>aiiA</i> gene from <i>Bacillus</i> sp. A24 under the constitutive P _{lac} promoter	47
pCR2.1	TA cloning vector, Amp ^r	Invitrogen
pKNOCK-Tc	Suicide vector for insertional mutagenesis; R6K <i>ori</i> Rp4 <i>oriT</i> Tc ^r	48
pRK600	Contains <i>tra</i> genes for mobilization, Chl ^r	49
pCR <i>hcnABC</i>	1.6-kb <i>hcnABC</i> fragment in pCR2.1	This study
pKNOCK- <i>hcnABC</i>	1.6-kb fragment from <i>hcnABC</i> in pKNOCK-Tc	This study
pUCP23	Broad-host-range vector, Amp ^r Gm ^r	50
pRSET-B mCherry	mCherry expression vector, <i>fl ori</i> , Amp ^r	51
pMCh-23	pUCP23 carrying the mCherry red fluorescent protein gene	18
Primers		
<i>hcnA</i> -FOR	5'-ATGGGCGTATGCCACTGC-3'	This study
<i>hcnC</i> -REV	5'-TAAGCACACGACGCGCCG-3'	This study
<i>sclerosin</i> -FOR	5'-CCACAAACGGCATTTTGCTGG-3'	This study
<i>sclerosin</i> -REV	5'-AGTTTGCTAAGGACCGCTGC-3'	This study
<i>hcnC</i> -FOR	5'-TACGTGGCGCAGAAAGACAACG-3'	This study
<i>hcnC</i> -REV	5'-TTGCACCAACCCCTTCGATCTCG-3'	This study
<i>pdfR</i> -FOR	5'-AGCATCATCGCCAACCAACACC-3'	This study
<i>pdfR</i> -REV	5'-GTTTTTCCCACTGCGAGCCAG-3'	This study
<i>pdfI</i> -FOR	5'-ACCGTTGACAGACGCAATATCG-3'	This study
<i>pdfI</i> -REV	5'-AGCGTTCTTGCTAAGGACCTCC-3'	This study
<i>rfa</i> -FOR	5'-GCACCTGAACCTGCCGAACAAC-3'	This study
<i>rfa</i> -REV	5'-GCATCCATCGGATAAGCGAACG-3'	This study
<i>gacS</i> -FOR	5'-TGGTGCAAACCCCTGCTCGAAG-3'	This study
<i>gacS</i> -REV	5'-TCTGCACGTCCATCAACACCAG-3'	This study

^a Rif^r, rifampin resistance; Tc, tetracycline; Gm^r, gentamicin resistance; Amp^r, ampicillin resistance; Chl^r, chloramphenicol resistance; Carb, carbenicillin.

culture (optical density at 600 nm [OD₆₀₀], 0.1; M9-Glc), which was incubated at room temperature for 72 h with shaking. A 1-ml volume of culture was spun for 1 min at 1,000 × g to pellet the nematodes, after which 400 μl of the bacterial cell suspension was added to 800 μl of RNeasy minikit (Qiagen, Valencia, CA, USA). Total RNA was extracted using the RNeasy minikit (Qiagen), and residual genomic DNA was removed by treatment with Turbo RNase-free DNase I (Ambion, Carlsbad, CA, USA).

cDNA was generated by reverse transcription using the Maxima first-strand cDNA synthesis kit (Thermo Scientific, Rockford, IL, USA) under the following conditions: initial heating at 25°C for 10 min, 50°C for 15 min for reverse transcription, and 85°C for 5 min for enzyme denaturation. The primer sequences for the genes of interest are listed in Table 1. Reverse transcription-quantitative PCR (qRT-PCR) was performed using a CFX Connect real-time system (Bio-Rad, Ontario, Canada) and SsoFast

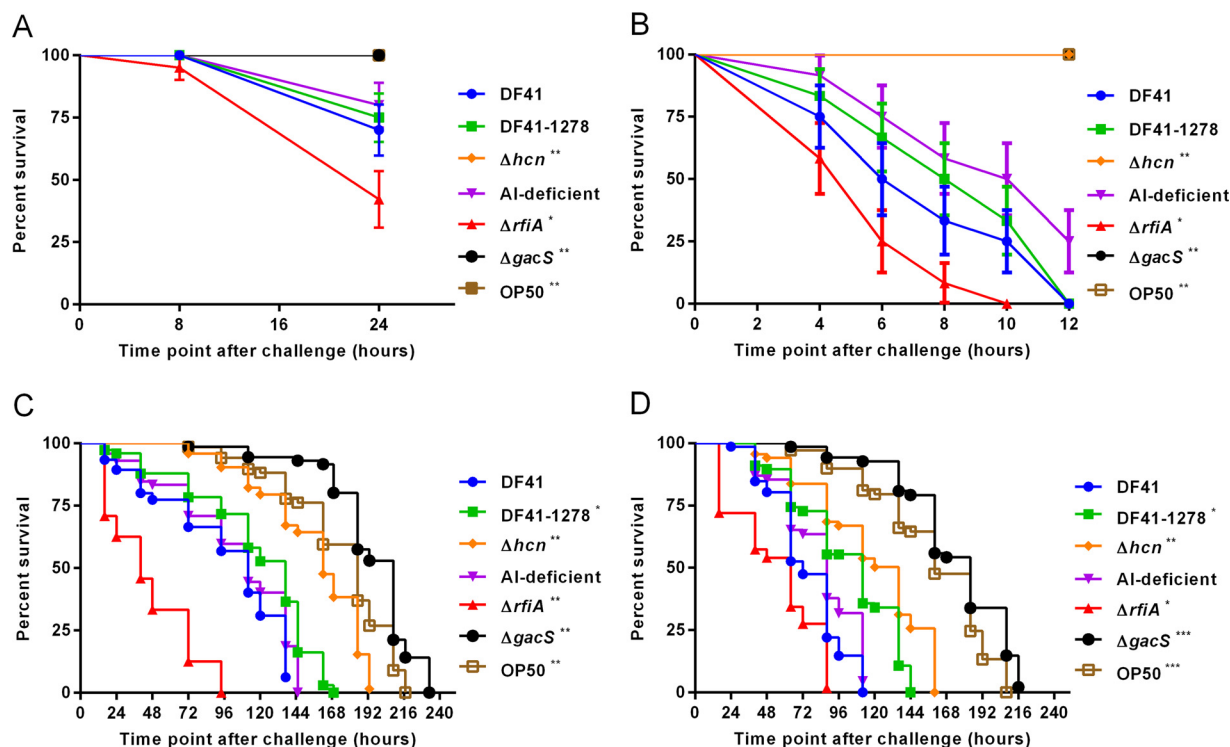


FIG 1 Fast-killing (A and B) and slow-killing (C and D) of *Caenorhabditis elegans* by *Pseudomonas brassicacearum* DF41. Kaplan-Meier survival plots of *C. elegans* N2 ($n = 25$) fed *E. coli* OP50, DF41, or derivative strains propagated on BHI agar (A), BHI agar supplemented with 100 μM FeCl_3 (B), NGM agar (C), and KB agar (D). Each data point represents the average of three biological replicates; error bars indicate the standard deviation (SD). Experiments were performed three times; one representative data set is shown. Asterisks indicate significant difference from the wild type as determined by the log rank test (*, $P < 0.01$; **, $P < 0.001$; ***, $P < 0.0001$). Note that in panels A and B, symbols representing the OP50, Δhcn mutant, and $\Delta gacS$ mutant strains overlap.

EvaGreen Supermix (Bio-Rad). The PCR conditions included an initial denaturation at 98°C for 2 min, followed by 40 cycles of 98°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Reactions were performed in triplicate, and experiments were repeated three times with four biological replicates. Relative gene expression was determined using the comparative threshold cycle (C_T) method, as described by Livak and Schmittgen (28).

Analysis of HCN and AHL signal production. DF41 was grown in the presence and absence of *C. elegans* for 72 h at room temperature on M9-Glc agar plates sealed with Parafilm. HCN production was monitored using Cyantesmo paper, which turns blue in the presence of this volatile compound. The production of AHL molecules was assessed qualitatively by spotting 5 μl of culture grown for 72 h in the presence and absence of *C. elegans* onto plates seeded with *Chromobacterium violaceum* CVO26. This bacterial strain is able to produce the purple pigment violacein only in the presence of exogenous autoinducer (29). Samples were analyzed in triplicate, and the experiments were repeated twice.

Statistical analysis. An unpaired Student t test was used for statistical analysis of biofilm formation, qRT-PCR gene expression, and AHL production. The log rank (Mantel-Cox) test was applied for statistical analysis of pairwise comparisons in the fast and slow nematode killing assays. Nematodes that died from crawling off the plate were excluded from statistical analysis.

RESULTS

***Pseudomonas brassicacearum* DF41 kills *Caenorhabditis elegans* through production of toxic metabolites.** When DF41 and derivative strains, including DF41-1278, DF41 hcn , autoinducer (AI)-deficient DF41, DF41 $rfiA$, and DF41 $gacS$, were grown on BHI, which supports fast killing by pseudomonads (7, 8), dif-

ferences in lethality were observed; however, none elicited 100% nematode killing at 24 h (Fig. 1A). The lowest level of toxicity was exhibited by the $gacS$ and hcn mutants. The sclerosin-deficient strain, DF41-1278, was similar to wild type, indicating that this lipopeptide (LP) does not contribute to fast killing. Similarly, the autoinducer (AI)-deficient DF41 strain (pME6863) exhibited no change in lethality. The most toxic strain was the $\Delta rfiA$ mutant. HCN production was increased in this background compared to the DF41 wild type (data not shown), likely accounting for the high nematocidal activity.

Although DF41 produces HCN, it does so at levels lower than what we have observed for other biocontrol strains, including *Pseudomonas chlororaphis* PA23 and *Pseudomonas protegens* Pf5 (data not shown). Supplementing media with iron has been reported to elevate HCN production (30); therefore, BHI containing 100 μM FeCl_3 was employed in the aforementioned assays to see if more rapid killing would ensue. While the same relative toxicity pattern was observed, the presence of iron did enhance overall lethality (Fig. 1B). When nematodes were placed on BHI (100 μM FeCl_3), the DF41 wild-type and sclerosin mutant strains caused 100% killing after 12 h. As before, the $\Delta rfiA$ mutant strain was the most toxic, killing 100% of the nematodes by 10 h, while the HCN-deficient hcn and $gacS$ mutants were completely benign (Fig. 1B). Collectively, these findings indicate that on BHI (100 μM FeCl_3), HCN is the primary compound responsible for fast killing of *C. elegans*. Due to reduced production of this volatile

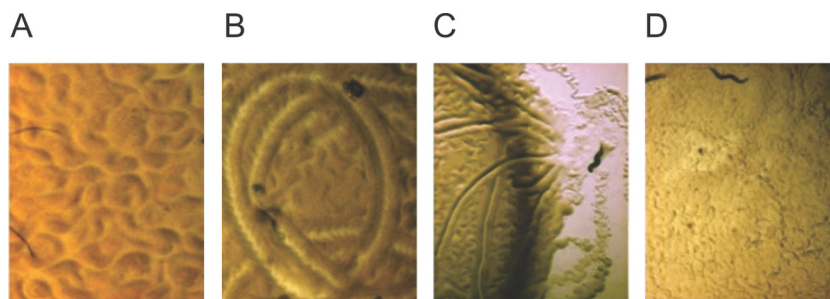


FIG 2 Track marks of nematodes fed on *E. coli* OP50 (A), DF41 (B and C), and a DF41 *gacS* mutant (D).

compound on media containing low FeCl_3 , however, rapid intoxication does not occur.

Slow killing of *Caenorhabditis elegans* by biofilm-dependent and biofilm-independent mechanisms. For the slow-killing assays, DF41 and mutant strains were grown on NGM and KB agar prior to seeding with nematodes. On NGM agar, the survival of the nematodes decreased over the course of days (Fig. 1C); survival rates were strain dependent but nevertheless consistent with lethality mediated by bacterial colonization rather than intoxication. Under these conditions, the *gacS* mutant was found to be less toxic than the *hcn* mutant. In addition to HCN, the *gacS* mutant fails to produce degradative enzymes and antibiotic compounds, including sclerosin (17), some of which may be contributing to toxicity. The sclerosin-deficient mutant (DF41-1278) showed reduced nematocidal effects compared to the wild type, indicating that this LP is involved in slow killing, albeit modestly. As before, the greatest toxicity was observed for the $\Delta rfiA$ mutant, which killed 100% of the nematodes within 96 h (Fig. 1C).

Slow killing of *C. elegans* was also observed when nematodes were fed bacteria growing on KB medium (Fig. 1D). However, there was one dramatic difference compared to nematodes fed DF41 and derivative strains grown on NGM. After 24 h, we noticed that biofilms began to form on the *C. elegans* head and body, which dramatically impacted nematode physiology and behavior (Fig. 2). Microscopic visualization at low magnification revealed that when nematodes were placed on NGM supplemented with the standard lab strain *E. coli* OP50, the nematodes moved freely through the bacterial lawns, generating sinusoidal track marks known as “skd” marks (Fig. 2A). Conversely, on DF41 lawns grown on KB medium, aberrant skd marks were formed (Fig. 2B and C), and the nematodes fishtailed through the viscous bacterial lawn like a car moving through deep snow. Moreover, the animals were frequently observed to migrate away from the bacteria, possibly in an attempt to remove their matrix entrapment (Fig. 2C). Over time, the biofilm-coated nematodes became progressively thinner, with some disintegrating completely, presumably due to starvation or exposure to toxic bacterial metabolites (data not shown).

DF41 biofilm formation on *Caenorhabditis elegans* is dependent on GacS. Next, we examined whether all of the DF41 derivative strains were capable of forming biofilms on the *C. elegans* head and body. On KB medium, the Δhcn , $\Delta rfiA$, sclerosin-lacking (DF41-1278), and AI-deficient mutant strains all coated the surface of *C. elegans* and resulted in aberrant skd marks (data not shown); however, *C. elegans* growth on and movement through the *gacS* bacterial lawn resembled those observed for *E. coli* OP50

(Fig. 2D). Confocal microscopic analysis of red fluorescent protein (RFP)-expressing bacteria revealed that, with the exception of the *gacS* mutant, all of the strains formed a thick biofilm on the *C. elegans* surface after 24 h (Fig. 3A). In all cases, bacteria are visible in the mouth, grinder, and intestinal track of the nematodes, indicating that bacterial ingestion had occurred prior to biofilm formation blocking the buccal cavity.

C. elegans mutants resistant to biofilm formation by *Y. pestis*, *Y. pseudotuberculosis*, and *X. nematophila* have been identified (13, 31, 32). To determine whether similar mechanisms of DF41 nematode attachment were at play, we examined several biofilm-resistant *C. elegans* strains, namely, the *srf-2(yj262)* and *bah-3(br9)* mutants and a naturally biofilm-resistant Hawaiian isolate (CB4856), growing on DF41. In all cases, biofilm formation was markedly reduced on the nematodes (Fig. 3B).

NaCl impacts DF41 biofilm development on the surface of *Caenorhabditis elegans*. The fact that biofilms developed on the nematode surface when bacteria were grown on KB, but not BHI, NGM, or LB, suggested that medium composition has an impact on this trait. One obvious difference in the composition of these media is salt content. The three media that did not promote biofilm formation contained NaCl (NGM [0.3%], BHI [0.5%], and LB [1%]), whereas KB medium does not. This finding prompted us to investigate whether NaCl affects the ability of DF41 to form biofilms on the surface of *C. elegans*. Nematodes fed DF41 grown on KB medium containing 0.5% NaCl resulted in a noticeable decrease in biofilm formation on the head and body (Fig. 4A). The effects were even more dramatic when the concentration of NaCl increased to 1%, in which case virtually no biofilms were observed on the nematodes (Fig. 4A).

Biofilm formation on abiotic surfaces is affected by NaCl but not GacS. Factors affecting attachment to abiotic surfaces are expected to be dramatically different from those involved in biofilm formation on biotic surfaces. As such, we employed a 96-well microtiter plate assay to assess the ability of DF41 and derivative strains to form adherent biomass under different medium conditions. While there was no significant difference in the ability of the strains to form biofilms on plastic, we did notice that in KB, biofilm formation was nearly double that observed for bacteria grown in NGM and LB (data not shown). These findings suggest that NaCl may be impacting biofilm formation not only on biotic surfaces but abiotic substrates as well. To test this hypothesis, we examined the adherent DF41 biomass after 24 h of static growth in KB supplemented with NaCl (0% to 1%). As illustrated in Fig. 4B, a significant decrease in biofilm formation was observed in KB containing 1% NaCl. No differences in growth rate were observed

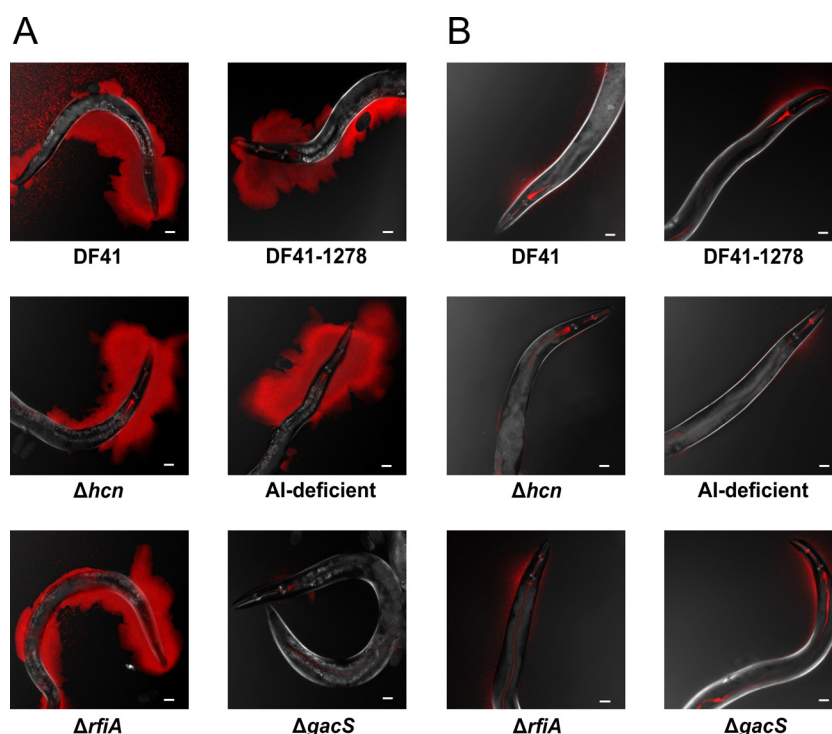


FIG 3 Microscopic analysis of N2 (A) and Δsrf -2 mutant (strain AT6) (B) worms fed *rfp*-tagged DF41 and derivative strains on KB. Confocal images were taken with Zeiss LSM 700 confocal laser scanning microscope under $\times 10$ magnification. Scale bar represents 20 μm .

when bacteria were grown in the presence of NaCl (data not shown); as such, reduced biofilm formation is not a consequence of altered growth rate.

DF41 gene expression is affected by growth in the presence of *Caenorhabditis elegans*. To investigate whether DF41 is able to sense the presence of *C. elegans*, either through direct contact or soluble chemical cues, we monitored bacterial gene expression upon growth in the presence and absence of these predators. Both regulatory genes (*gacS*, *pdfR*, *pdfI*, and *rfiA*) and the biosynthetic loci encoding HCN and sclerosin were analyzed. The most dra-

matic increase in gene expression was observed for *hcnA*, which was elevated over 5-fold in the presence of the nematodes; conversely, sclerosin biosynthetic gene activity was not affected (Fig. 5). Expression levels of the QS regulatory genes *pdfR*, *pdfI*, and *rfiA* were all significantly elevated upon *C. elegans* coculture (Fig. 5). Transcription of *gacS*, on the other hand, remained unaffected. We observed increased HCN and AHL signal production in DF41 cells cocultured with *C. elegans* (Fig. 6), consistent with the increase in *hcnA* and *pdfI* transcription, respectively.

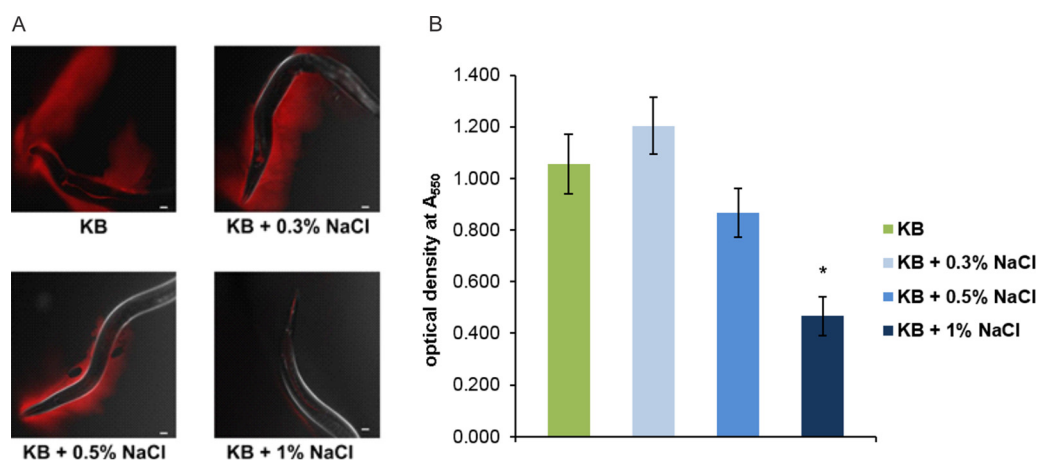


FIG 4 (A) Microscopic analysis of N2 worms fed *rfp*-tagged DF41 propagated on KB supplemented with NaCl (0 to 1%). Confocal images were taken with Zeiss LSM 700 confocal laser scanning microscope under $\times 10$ magnification. Scale bar represents 20 μm . (B) *In vitro* biofilm formation by DF41 in KB broth supplemented with NaCl (0 to 1%). Each value represents the mean from three biological replicates \pm standard error. The data point marked with an asterisk indicates statistical significance ($P < 0.01$).

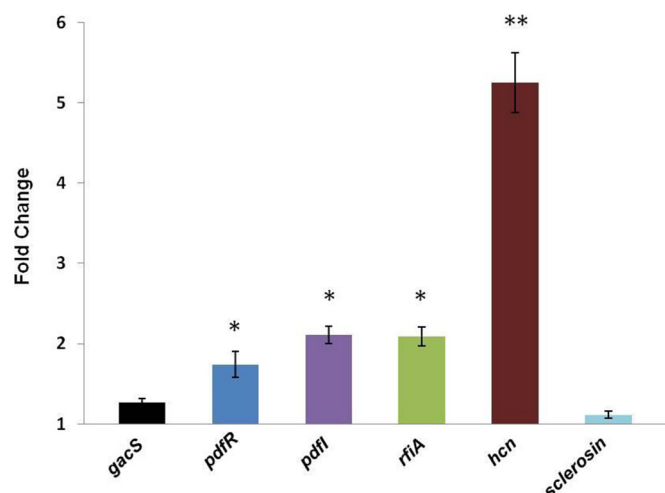


FIG 5 The impact of *C. elegans* coculture on DF41 biocontrol gene expression. qRT-PCR analysis was used to quantitatively assess gene expression in the presence and absence of nematodes. Expression levels in the absence of *C. elegans* were normalized to 1; differentially expressed genes are indicated with asterisks (*, $P < 0.05$; **, $P < 0.001$). Data shown are the means; error bars indicate SD.

DISCUSSION

In the current study, we explored the interaction between the biocontrol agent *P. brassicacearum* DF41 and the bacterivorous predator *C. elegans*. We discovered that DF41 is among a select group of bacteria capable of establishing biofilms on the *C. elegans* surface, blocking feeding and ultimately starving the nematodes. To the best of our knowledge, biofilm formation on *C. elegans* has never been reported for a pseudomonad, pathogenic or otherwise.

It is well established that medium composition has a profound impact not only on secondary metabolites produced by pseudomonads (33) but also the nematocidal activity associated with these organisms (5–8, 34). Consequently, several different media were employed as part of our DF41-*C. elegans* interaction studies. On nutrient-rich BHI, which has been previously reported to support high exometabolite production and fast killing of *C. elegans* (6, 7), none of the strains tested led to 100% killing at 24 h (Fig. 1A). Because HCN is a nematocidal agent causing rapid paralysis of *C. elegans* (6, 8), we hypothesized that HCN levels were below those required for fast killing. When FeCl_3 was added to the media to boost HCN production, the rate of killing by DF41 was enhanced such that all of the nematodes were dead by 12 h (Fig. 1B). With respect to relative toxicity, both in the presence and absence of FeCl_3 , the strain exhibiting the highest level of nematocidal activity was the *rfiA* mutant (Fig. 1A and B). The *rfiA* mutant exhibits increased accumulation of AI molecules and elevated expression of QS-controlled genes, including *pdfI* and *hcnA* (20). HCN analysis revealed elevated production of this volatile by the *rfiA* mutant (data not shown), which likely accounts for the increased nematocidal activity. We cannot rule out the possibility that yet-to-be identified metabolites trapped within these cells contribute to toxicity. HCN is clearly an important toxin, since the Δhcn and $\Delta gacS$ mutant cells, which are both devoid of HCN production (17), show significantly reduced toxicity, consistent with previous findings (6, 8). Sclerosin, on the other hand, did not contribute to killing under these conditions.

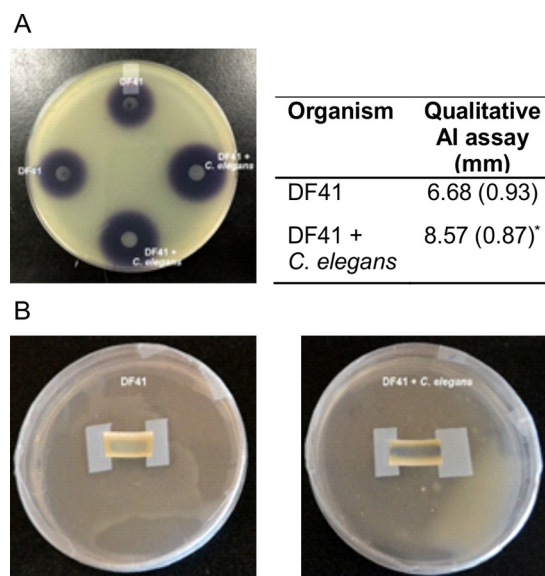


FIG 6 (A) Autoinducer production by DF41 grown in the presence and absence of nematodes detected by the AHL biosensor *Chromobacterium violaceum* CVO26. The mean (SD) of zones of activity (in millimeters) obtained from 10 replicates are shown in the table. The asterisk indicates a significant difference from the wild type ($P < 0.001$). (B) HCN production by DF41 grown in the presence and absence of *C. elegans*.

In slow-killing assays, which depend upon infection of the *C. elegans* intestine, killing occurred over the course of days (Fig. 1C and D). On both NGM and KB media, HCN, and to a lesser degree, sclerosin, contributed to the nematocidal effects; however, one dramatic difference was observed between the two media. After 24 h on KB, biofilms began to accumulate on the surface of the nematodes as they translocated through the bacterial lawns. The deleterious effect of biofilm formation is reflected by the fact that with the exception of the *gacS* mutant and OP50, which do not form biofilms, *C. elegans* survival decreased on bacteria propagated on KB (Fig. 1D).

In *Y. pestis*, *Y. pseudotuberculosis*, and *X. nematophila*, biofilm development on *C. elegans* is dependent upon the *hmsHFRS* gene cluster (11, 32). Analysis of the DF41 genome revealed the presence of a homologous *hmsHFRS* operon (*hmsH*, CD58_RS00620; *hmsF*, CD58_RS00625; *hmsR*, CD58_RS00630; and *hmsS*, CD58_RS00635) (35). This cluster showed the highest degree of similarity at the amino acid level with *PgaABCD* of *Pseudomonas fluorescens*. The DF41 HmsH protein is 91% identical to PgaA, a predicted poly-beta-1,6-*N*-acetyl-D-glucosamine (PGA) export porin (accession no. ALI07281.1). HmsF exhibits 94% sequence identity with PgaB, a PGA *N*-deacetylase (accession no. WP_024618876.1). HmsR is 99% identical to the PGA synthase PgaC (accession no. ALI07279.1), and HmsS exhibits 93% identity with the PGA biosynthesis protein PgaD (WP_003186997.1). In *E. coli*, PGA biosynthesis mediated by *pgaABCD* is essential for biofilm formation (36, 37). BLAST analysis revealed that homologs of the *hmsHFRS* operon are found in only a limited number of *Pseudomonas* species (Table 2). It is noteworthy that these genes are absent in *P. aeruginosa*, which does not form biofilms on *C. elegans* (15). Conversely, biocontrol strain *P. chlororaphis* PA23, which harbors this locus, was unable to form biofilms on the nematode surface under the conditions tested (data not

TABLE 2 Sequence identity of DF41 HmsHFRS with homologs found in other *Pseudomonas* species

Bacterial strain	Amino acid identity (%) of homolog to DF41 HmsHFRS
<i>P. chlororaphis</i> strain PA23	78
<i>P. koreensis</i> strain D26	80
<i>P. poae</i> strain RE*1-1-14	74
<i>P. antarctica</i> strain PAMC 27494	74
<i>P. azotoformans</i> strain S4	75
<i>P. simiae</i> strain WCS417	74
<i>P. trivialis</i> strain IHBB745	74
<i>P. citronellolis</i> strain P3B5	59

shown). Additional work is required to establish a role for these genes in DF41 biofilm development; however, the fact that *C. elegans* mutants that are resistant to *Yersinia* and *Xenorhabdus* biofilms are resistant to those of DF41 suggests that a common ligand-receptor interaction is involved (15, 32).

In *E. coli*, a *csrA* (*rsmA*) mutant was found to overproduce PGA and showed increased biofilm development (38, 39). CsrA belongs to the RsmA family of repressor proteins that bind mRNA at the ribosome-binding site, thereby blocking translation (40). The Gac two-component system is a positive activator of small RNAs that titrate out the RsmA proteins allowing target gene expression. Consequently, in a *gac* mutant, targets remain permanently repressed. The findings presented herein suggest that genes underlying DF41 biofilm formation on the surface of *C. elegans* are subject to Gac regulation, consistent with the CsrA control observed in *E. coli*. In another study, the LysR-type regulator NhaR was found to activate *pgaABCD* expression in response to 100 mM NaCl and high pH in *E. coli* (41). We, on the other hand, observed that the addition of sodium ions (0.5% [86 mM] and 1% [171 mM]) diminished the establishment of DF41 biofilms on both biotic and abiotic surfaces (Fig. 4). A negative correlation between NaCl and biofilm formation has been reported for *Clostridium difficile* and *Streptococcus suis* (42, 43). Collectively, these findings suggest that the impact of sodium on biofilm development is species dependent.

Prokaryotes and eukaryotes have cohabited the Earth for millions of years; it is not surprising, therefore, that chemical communication between the two facilitates mutual perception (44). The production of toxic secondary metabolites is energetically costly, and so limiting production to situations where these compounds provide a fitness advantage, when confronted by predators, for example, would be beneficial to the producer. We cocultured DF41 in the presence of *C. elegans* to see whether chemical cues and/or direct contact would lead to changes in the expression of select bacterial genes. Upon coculture, the most highly upregulated DF41 gene was *hcnA* (Fig. 5). Intriguingly, HCN also exhibited the greatest nematocidal activity (Fig. 1). Sclerosin, which exhibited almost no toxicity toward *C. elegans*, was unchanged with respect to gene expression in DF41 grown with the nematodes. These findings are similar to those reported by Jousset and co-workers, wherein cell-free supernatants of the amoeba *Acanthamoeba castellanii* were found to increase the expression of *phlA* (diacetylphloroglucinol [DAPG]), *prnA* (pyrrolnitrin), and *hcnA* in *P. fluorescens* CHA0 (22). Notably, there was a correlation between the level of expression and the toxicity of the encoded prod-

uct, with the greatest increase in gene expression observed for DAPG, the most toxic metabolite tested (22). More recently, global transcriptomic analysis of *P. fluorescens* SS101 cocultured with the protist *Naegleria americana* revealed altered expression of 2.3% of the genome (2). Moreover, protozoan predation led to upregulated lipopeptide and putrescine biosynthesis, with the putrescine biosynthesis inducing trophozoite encystment and decreasing cyst viability (2). When we examined the impact of coculturing on DF41 regulatory gene expression, we discovered that *pdfR*, *pdfI*, and the cotranscribed *rflA* were upregulated in the presence of *C. elegans* (Fig. 5). We have previously shown that *hcn* expression is under the control of the PdfRI QS system, whereas the sclerosin biosynthetic genes are not (20). At present, it is unclear whether upregulation of the *hcn* genes is mediated directly or indirectly through increased QS gene expression. Analysis of HCN and AHL signal production by DF41 revealed that both end products were upregulated by the presence of the nematodes, consistent with our gene expression analysis.

Being encased in an extrapolymeric matrix as a biofilm promotes bacterial survival and colonization of root surfaces (45). In addition to facilitating exchange of metabolic by-products and genetic information, this mode of growth affords inhabitants protection from toxic compounds and desiccation. We have made the exciting discovery that DF41 is capable of biofilm formation on the *C. elegans* anterior, blocking feeding and ultimately starving the animals. Thus, for DF41, biofilm formation offers protection from grazing predators. It is possible that biocontrol agents that show increased environmental persistence through biofilm formation do so through similar antipredator strategies. As we unravel the biotic and abiotic factors underlying this phenomenon, transkingdom signaling between plants, bacteria, and the predators that feed upon them is expected to play a central role.

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