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BACTERIAL ATTRACTION AND QUORUM SENSING INHIBITION IN *CAENORHABDITIS ELEGANS* EXUDATES

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Abstract

Caenorhabditis elegans, a bacterivorous nematode, lives in complex rotting fruit, soil, and compost environments, and chemical interactions are required for mating, monitoring population density, recognition of food, avoidance of pathogenic microbes, and other essential ecological functions. Despite being one of the best-studied model organisms in biology, relatively little is known about the signals that *C. elegans* uses to chemically interact with its environment or as defense. *C. elegans* exudates were analyzed using several analytical methods and found to contain 36 common metabolites including organic acids, amino acids and sugars, all in relatively high abundance. Furthermore, the concentrations of amino acids in the exudates were dependent on developmental stage. The *C. elegans* exudates were tested for bacterial chemotaxis using *Pseudomonas putida* (KT2440), a plant growth promoting rhizobacterium, *Pseudomonas aeruginosa* (PAO1), a soil bacterium pathogenic to *C. elegans*, and *E. coli* (OP50), a non-motile bacterium tested as a control. The *C. elegans* exudates attracted the two *Pseudomonas* species, but had no detectable antibacterial activity against *P. aeruginosa*. To our surprise, the exudates of

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Author contributions- FK and DVB contributed equally and led the study; FK, ATD, RA, HA, and MS intellectually contributed to the worm exudate protocol which was developed in the ASE laboratory; FK, RA, and RN collected exudates; DVB conducted bacterial bioassays; HA collected LC-MS data; FK and CZ collected NMR data; FZ, SLR and RB did COLMAR analysis; FK, CZ, MS, and ATD manually analyzed NMR data. SR and FS analyzed amino acids by HPLC; LHL analyzed young adult exudates by GC-MS; WZ did principle component analysis; FK, DVB, ASE and JMV analyzed the data and wrote the paper with help from the entire team.

young adult and adult life stages of *C. elegans* exudates inhibited quorum sensing in the reporter system based on the *LuxR* bacterial quorum sensing (QS) system, which regulates bacterial virulence and other factors in *Vibrio fischeri*. We were able to fractionate the QS inhibition and bacterial chemotaxis activities, demonstrating that these activities are chemically distinct. Our results demonstrate that *C. elegans* can attract its bacterial food and has the potential of partially regulating the virulence of bacterial pathogens by inhibiting specific QS systems.

Keywords

C. elegans exudates; bacterial chemotaxis; metabolomics; quorum sensing inhibitor; chemical ecology

INTRODUCTION

C. elegans was the first metazoan to have its genome sequenced (Wilson et al., 1994) and has one of the best annotated animal genomes (www.wormbase.org). Its entire cell lineage from a single fertilized egg to an adult is known and has been related to the animal's anatomy (Sulston et al., 1983), and its anatomical ultrastructure has been comprehensively described by thin-section electron microscopy (White et al., 1986). *C. elegans* is particularly tractable for genetic studies (Brenner, 1974), and as a result many signal transduction pathways have been identified. Several biomedically important discoveries have been made using *C. elegans* including apoptosis (Yuan et al., 1993; Ellis et al., 1991), RNAi (Fire et al., 1998), and the *in vivo* application of the green fluorescent protein (Chalfie et al., 1994). In short, *C. elegans* is one of the best-understood animals in science. Natural *Caenorhabditis* species can be isolated from compost heaps, soil, decaying fruits, and even carrier invertebrates (Barriere and Felix, 2006). Despite the wealth of genetic, cellular, and anatomical information, relatively little is known about *C. elegans* in its natural environment.

Small molecules are the primary mode of communication for most organisms. Bacteria, fungi, and plants release a variety of chemicals that attract or deter other organisms (Tso and Adler, 1974; Adler, 1966; Mesibov and Adler, 1972; Hedblom and Adler, 1983; Adler et al., 1973; Bacilio-Jiménez et al., 2003; Kumar et al., 2007; Lugtenberg et al., 1999; Singh and Arora, 2001; van der Drift et al., 1975). Plant roots produce exudates that are capable of killing microbes, and these antimicrobial exudates can be up-regulated in response to pathogens (Walker et al., 2004; Bais et al., 2005). Several species of bacteria release acyl homoserine lactones to determine their local cell density (quorum sensing - QS), activate virulence factors at high cell density, and aggregate into biofilm (Miller and Bassler, 2001; Williams et al., 2007). Some bacterial QS signals have been shown to attract *C. elegans* (Beale et al., 2006). Some species of plants (Givskov et al., 1996; Persson et al., 2005) and fungi (Rasmussen et al., 2005b) produce compounds that inhibit bacterial QS. Similar to bacteria, *C. elegans* also sense their own population density *via* dauer pheromones (Golden and Riddle, 1982), which are ascaroside sugars with modified fatty acid groups (Jeong et al., 2005; Butcher et al., 2008; Srinivasan et al., 2008; Butcher et al., 2007). These pheromones regulate the formation of a specific larval stage called dauer by integrating chemical cues

from both the pheromone and an unknown chemical cue from their bacterial food source (Golden and Riddle, 1982). *C. elegans* hermaphrodites also produce a chemical cue that attracts males (Simon and Sternberg, 2002; White et al., 2007), and this mating cue was recently discovered to consist of a synergistic mix of known dauer pheromones called ascr#2 and ascr#3 (Butcher et al., 2007) and a glycosylated analogue called ascr#4, which is active at concentrations much lower than that required for dauer formation (Srinivasan et al., 2008). Recently, additional *C. elegans* ascarosides have been discovered with both dauer and mating activities (Pungaliya et al., 2009). Despite the extensive recent research into *C. elegans* chemical signaling, relatively little is known about how *C. elegans* chemically interacts with microorganisms in its environment or even its natural food (Shtonda and Avery, 2006; Avery and Shtonda, 2003).

In this study, we present a comprehensive analysis of the chemical composition of *C. elegans* exudates using several analytical techniques, which allowed the identification of several amino acids, sugars, and organic acids that are released by the nematode into its environment. The majority of these chemicals are amino acids, and we present a quantitative analysis of changes in amino acid concentration as a function of development. The *C. elegans* exudates contain several compounds that have been shown to cause bacterial chemotaxis, and we show that at least two species of bacteria, including the pathogenic *Pseudomonas aeruginosa*, are attracted to the exudates. This observation suggested the possibility that *C. elegans* exudates may employ a mechanism to help protect themselves from pathogens. We found that *C. elegans* exudates produce a compound that inhibits quorum sensing in the reporter system based on the *LuxR* quorum sensing system (Andersen et al., 2001; Rasmussen et al., 2005a), and this compound is developmentally regulated and distinct from the compounds that cause bacterial chemotaxis. These results suggest a rich and complex set of chemical interactions between *C. elegans* and its environment.

METHODS AND MATERIALS

Sample Preparation

C. elegans exudates were collected at defined life stages (L1, L2, L3, L4, young adult and adult). Synchronized *C. elegans* (N2 Bristol) with a worm density of ~10,000 worms/ ml were co-cultured with *E. coli* (strain HB101) at 22°C at 250 rpm on S-complete medium. The worms were harvested and separated from the medium and *E. coli* by centrifugation or gravity followed by rinsing three times over a nylon filter. They were then allowed to digest bacteria in their gut for 30 min in M9 buffer, rinsed three times in water, and incubated in water for 1 hr at density of ~30,000 worms/ ml, as previously described (Jaffe et al., 1989; Stiernagle, 1999; Johnstone, 1999; Srinivasan et al., 2008). We were recently able to isolate and identify the *C. elegans* mating pheromone using a similar preparation with the same relatively high worm density (Srinivasan et al., 2008). The primary purpose of the high density of worms is to efficiently collect enough material for efficient analytical characterization. All of the quantitative results shown below are normalized to the material produced by one worm in one hour (defined as 1 worm equivalent: WE). Therefore, the density of the worm culture will not affect the outcome unless the exudates are themselves dependent on worm density. To rule out the possibility that the chemicals might be coming

from dead worms, they were checked under the light microscope to verify whether they were alive before and after they were placed in water. As a negative control, we also made a similar preparation with only *E. coli* HB101 without worms. It should be noted that we are unable to distinguish whether the exudates were from regulated secretions or from defecation. The bacterial controls without worms allow us to rule out bacteria as the direct source of the exudates. For almost all experiments described below, we used the same replicate sample preps, three at each developmental stage. The exception was for the data shown in Figure 4 in which we needed to produce additional replicates for C18 fractionation and bioassays.

NMR Data Collection from *C. elegans* Exudates

For each life stage, *C. elegans* exudates from two independent experiments were lyophilized, and each sample was separately dissolved in 25 μ l deuterium oxide (D_2O). A total volume of 12 μ l containing ~54,000 worm equivalents (WE) and 0.25 mM 3-(Trimethylsilyl)-propionic acid- D_4 , a proton chemical shift reference standard (TSP=0.0 ppm) was transferred to a 1 x 100 mm capillary NMR tube (Norell, Inc., NJ). NMR spectra of worm exudates were collected at 600 MHz on a Bruker Avance II 600 console in a 14.1 T magnet, using a 1-mm triple resonance high temperature superconducting (HTS) cryogenic probe (Brey et al., 2006). The NMR data were collected with a sample temperature of 300 K and spectral width of 7211.53 Hz. The carrier frequency of 1H was centered on residual water, which was eliminated by presaturation or by gradient methods. Two-dimensional total correlation spectroscopy (TOCSY) data were collected using the DIPSI-2 mixing sequence (Shaka et al., 1988) with 60 or 90 ms mixing time, 2048 complex data points along the direct and 1024 or 512 complex data points along the indirect dimension, respectively. TOCSY data were processed using NMRPipe (Delaglio et al., 1995) by eliminating residual water by deconvolution, followed by apodization using a cosine squared function, zero-filled two times, Fourier transformed, and baseline corrected along both the dimensions. Data were analyzed and assigned with NMRview (Johnson, 2004). To manually identify known metabolites by NMR, TOCSY spectra were overlaid with spectra of standard compounds downloaded from the BMRB metabolomics database (Markley et al., 2007). To confirm the different compounds identified in worm exudates, the samples were spiked with 1 to 2 mM of the respective authentic compound in D_2O , followed by the acquisition of both 1D and 2D NMR spectra.

Semi-automated Compound Identification by NMR

Chemical constituents were identified using the webserver-based COLMAR protocol (Robinette et al., 2008), which provides processing and analysis of 2D NMR spectra of metabolic mixtures (<http://spinportal.magnet.fsu.edu>). The 2D TOCSY data were processed using a covariance algorithm (Brüschweiler and Zhang, 2004; Trbovic et al., 2004) that yields spectra with equally high resolution along both frequency dimensions. The covariance TOCSY spectra were then deconvoluted by COLMAR DemixC (Zhang and Brüschweiler, 2007; Zhang et al., 2007), which extracts 1D spectral traces that represent individual compounds by identifying spin systems with minimal likelihood of overlaps between different compounds in the covariance TOCSY spectra of the intact mixtures. In the final step, chemical shifts from individual spin systems derived from the DemixC traces were

screened against the BMRB metabolomics spectral database (Seavey et al., 1991) using our COLMAR query webserver (Robinette et al., 2008; Snyder et al., 2008), which outputs a ranked list of the highest scoring compounds.

Gas Chromatography and Mass Spectrometry (GC-MS) Analysis

To increase the number of identified compounds and to verify automated compound identification by NMR, we used GC-MS. We lyophilized 1 ml of young adult exudates (30,000 WE/ ml) and *E. coli* (HB101) controls from three independent experiments, and chemically derivatized the lyophilized material using a two-step approach (Fiehn et al., 2000; Roessner et al., 2000; Wagner et al., 2003). Forty μ l of 20 mg/ml methoxyamine hydrochloride in pyridine containing ribitol as an internal standard was directly added to the dried samples and vigorously mixed at 30°C for 1.5 h. Subsequently, 80 μ l of N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) containing a mixture of C₁₂, C₁₅, C₁₉, C₂₂, C₂₈, C₃₂, C₃₆ n-alkanes was added and incubated at 37°C for 30 min. Derivatized samples were immediately analyzed by TRACE DSQ GC-MS system (Thermo Finnigan Corp. Austin, TX, USA). Chromatograms were analyzed using the software AMDIS (version 2.64) in retention time index (RI) calibration mode. Metabolite identification was achieved by searching a user-library from the Max Planck Institute of Molecular Plant Physiology, Golm, Germany (Dr. Joachim Kopka, personal communication) that contains both retention time index and electron impact (EI) mass spectrum for each target component. Compounds were considered identified if their matching factor was greater than 800 on a scale of 0-1000 and RI deviation was less than 3.0. Quantitative information for each component was extracted from the chromatogram by using the AMDIS software and a customized MACRO (<http://www.lssc.nasa.gov/als/chemistry>, then view features → metabolomics tool) and subsequently normalized to ribitol's response.

Quantification of Amino Acids by High Performance Liquid Chromatography (HPLC)

To quantify the concentrations of amino acids present in worm exudates, an HPLC fluorescent detection methodology (Cohen and Michaud, 1993) was employed. Three independent replicates of 3 ml of exudates at each developmental stage were lyophilized and resuspended in 150 μ l water. To make the amino acids fluorescent, samples were derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC, AccQ•Fluor™ Reagent Kit, Waters, Milford, MA) following the manufacturer's protocol with minor modifications as indicated below. Reconstituted exudates (2.5 μ l) were mixed with 17.5 μ l of AccQ•Fluor borate buffer followed by the addition of 5 μ l of AccQ•Fluor Reagent. The resulting mixture was incubated at 55°C for 10 min in a heating block. Prior to HPLC separation, the derivatized samples were diluted to 50 μ l with water and were filtered through a 0.22- μ m PVDF membrane.

The resulting fluorescent derivatives were separated on a SunFire C₁₈ column (4.6 x 150 mm, 3.5 μ m) or a Nova-Pak C₁₈ column (3.9 x 150 mm, 4.0 μ m) using a Waters Alliance 2695 separation module and were measured by a Waters 2475 fluorescence detector using an excitation wavelength of 250 nm and an emission wavelength of 395 nm. Two separation procedures under gradient conditions (Supplementary Tables S2 and S3) were used to ensure accurate identification and quantification of amino acids. Solvent composition

consisted of A, water; B, acetonitrile; C1, 140 mM sodium acetate and 17 mM triethylamine, pH 5.05; C2, 100 mM sodium acetate and 5.6 mM triethylamine, pH 5.7; D, 100 mM sodium acetate and 5.6 mM triethylamine, pH 6.8. The volume of sample injected was 10 μ l. The AQC-amino acid adducts were identified and quantified by comparison with standards.

Liquid Chromatography and Mass Spectrometry (LC-MS) Analysis

To search for specific known metabolites, we utilized LC-MS. One ml (30,000 WE) of worm exudates from defined developmental life stages was lyophilized and then dissolved in 50 μ l of methanol. Ten μ l were analyzed using a Thermo Finnigan LCQ Deca XP Max with electrospray ionization in positive and negative ion modes. Additionally, 1 nmol of commercially available patulin (MP Biomedicals, Ohio USA) and penicillic acid (Acros organic, New Jersey, USA) were analyzed under the same conditions as above to establish retention times and diagnostic ions for screening worm exudates for these compounds.

Principle Component Analysis (PCA)

We used PCA to cluster exudates from different larval stages using measurements of 14 amino acids that were quantified by HPLC. The concentration ranges of amino acids in worm exudates vary greatly, both between different amino acids and between the different *C. elegans* life stages for a given amino acid. For example, the concentration of Met ranges from about 0 to 0.19 pmol/ WE, while Ala ranges between about 0 and 6 pmol/ WE. When data are not properly scaled, the performance of PCA is dominated by high concentration metabolites, because they constitute most of the variance. To give each metabolite an equal weight in the analysis, we scaled the data by first subtracting the mean from each metabolite and then dividing by root-mean-square. The scaled metabolite measurements have mean 0 and variance 1. PCA analysis (Johnson and Wichern, 2001) was performed on the scaled data using R software (<http://www.r-project.org/>).

Chemotaxis Assay

A slightly modified agarose plug method (Yu and Alam, 1997) was used to test for bacterial chemotaxis activity of worm exudates. Briefly, bacteria grown in LB media overnight were used as a starter culture and inoculated again in the fresh LB at a dilution of 1:50. Rifampicin (20 μ g/ml) was added to *P. aeruginosa* (PAO1), because this strain is resistant to that antibiotic. No antibiotics were added to *P. putida* (KT2440) and *E.coli* (HB101) cultures. This culture was placed in a shaker incubator until reaching an OD₆₀₀ of 0.4 – 0.6, the cell density corresponding to the maximum proportion of motile cells. The cells were collected by centrifugation, washed twice with chemotaxis buffer (10 mM potassium phosphate, pH 7.0, 0.1 mM EDTA, 1 mM MgSO₄) and resuspended to an OD₆₀₀ of 0.1 for the chemotaxis assay. A 10 μ l drop of 2% agarose solution made with chemotaxis buffer and 10 μ l of worm exudate (~300 W.E.) were mixed and placed on the center of the acetone cleaned microscopic slide, framed with two plastic strips (16 mm apart) and covered with a cover slip to create a chemotaxis chamber. The slide was allowed to stand for one minute to solidify the agarose plug. Once this was achieved, 120 μ l of bacterial suspension was added to the chamber surrounding the agarose plug and incubated at room temperature for 15–30

min. Following incubation, the distribution of cells immediately adjacent to the agarose plug was observed under a phase-contrast microscope and recorded with a charge-coupled device camera. Positive and negative controls were with agarose plugs containing just LB medium and chemotaxis buffer, respectively. For each trial, at least three agarose plugs were used for each developmental stage of *C. elegans* exudates. These experiments were conducted in triplicate with three independent worm exudate preparations.

Antimicrobial Assay

A modified agar diffusion method (Bauer et al., 1966) was used to test for antimicrobial activity of worm exudates against the opportunistic pathogen *Pseudomonas aeruginosa* (PAO1). Luria-Bertani (LB) (Bertani, 1951) agar plates were prepared and spread homogenously with 100 μ l of an overnight grown culture of *P. aeruginosa* (PAO1) at a concentration of 0.02 (10^7 cfu/ ml) suspended in 10 mM $MgSO_4$ buffer. The plates were dried for 5 min under a sterile hood. Thereafter, sterile Whatman filter paper discs, 7 mm in diameter, were placed on the surface of seeded agar plates and loaded with 50 μ l and 100 μ l of filter-sterilized exudates (~1,500 and 3,000 WE) and air dried. Sterile filter paper discs, loaded with 100 μ l of sterile distilled water, were used as a negative control. After the plates were incubated at 37°C for 16 h, they were examined for the presence of zones of bacterial growth inhibition. These experiments were conducted in triplicate.

Quorum Sensing (QS) Inhibition Assays

We used both a QSI1 reporter system (Rasmussen et al., 2005a) and a green fluorescent protein (GFP) based N-acyl homoserine-lactone (AHL) sensor system (*GFP-AHLs* sensor) (Andersen et al., 2001) to test for the QS inhibition (QSI) activity of worm exudates. Both reporter constructs are based on the LuxR system of *Vibrio fischeri* and are plasmids expressed in *E. coli* for efficient screening of QSI activity. Both assays were conducted in the presence of activating AHLs. In the QSI1 system, a toxic gene is expressed unless an inhibitor is added, so QSI activity is required for *E. coli* growth. In the GFP-based system, GFP is expressed with AHLs, and the amount of GFP expression and thus fluorescence is reduced with added QSI activity. The medium used for this assay was ABT minimal medium supplemented with 0.5% glucose and 0.5% Casamino acids (AB medium containing 2.5 mg of thiamine/L) (Clark and Maaole, 1967). Ampicillin (100 μ g/ml) was added to prevent contamination of the *E. coli* assay systems. A total of 50 μ l of exudate (~1500 WE) for QSI1 was tested. Additionally, 50 μ l of chamomile extracts and sterile water served as positive and negative controls, respectively, for QSI1.

For the *gfp-AHLs* sensor system, we used a microtiter dish and tested the QSI activity of worm exudates in the presence of 100 nM *N*-3-oxohexanoyl-L-homoserine lactone. Each well contained 100 μ l of test samples, 100 μ l of diluted bacterial culture (*E. coli* MT102 harboring pJBA132) in ABT medium and 100 nM *N*-3-oxohexanoyl-L-homoserine lactone. The final bacterial density (OD_{450}) was adjusted to 0.05. GFP fluorescence was monitored at an excitation wavelength of 475 nm and emission detection at 515 nm along with bacterial density at OD_{450} every 20 min for 7 h at 30 °C using a Synergy 4 microplate reader (BioTek, Winooski, VT). Sterile water and 12.5 μ M 4-nitropyridine N-oxide (4-NPO) (Sigma-Aldrich) were negative and positive controls, respectively. The test samples

included prefractionated young adult *C. elegans* exudates (~3000 WE), exudate fractions which were separated by C18 solid phase extraction into flow through, 50% methanol (MeOH), and 90% MeOH fractions (Srinivasan et al., 2008), and a reconstituted fraction in which the flow through, 50 and 90 % MeOH fractions were recombined.

QS-dependent Virulence Factor Assays in *P. aeruginosa*

We also used three assays—pyocyanin, elastase, and protease—to test for QSI activity of worm exudates in *P. aeruginosa*. The details of these assays methods and results are provided in the supplementary material.

RESULTS

Chemical Composition of *C. elegans* Exudates

We used three techniques—NMR spectroscopy, HPLC, and GC-MS—to identify the major compounds in worm exudates. These complementary techniques allowed us to identify 36 low molecular weight compounds including sugars, amino acids, and other organic acids (Table 1). Qualitative changes in exudates during *C. elegans* development can be seen in the 1D ¹H NMR spectra in Figure 1. These spectra show that worm exudates contain a relatively complex mixture of compounds, some of which are constant and some variable in concentration throughout development. The most striking changes were in methyl resonances with chemical shifts around 1 ppm (Fig. 1). These signals were strong from L2 to young adult, but significantly diminished in adult animals. Resonances with chemical shifts around 8.3 ppm, presumably from aromatic compounds, were present in adult exudates but not from other life stages.

Initial compound identification was achieved by overlaying TOCSY spectra of common metabolites downloaded from the BMRB metabolomics database. We also used COLMAR DemixC, a recently developed semi-automated method to analyze NMR spectra of complex mixtures (Zhang and Brüschweiler, 2007; Zhang et al., 2007). About 20% of the compounds could be identified using COLMAR, including sugars (glucose and trehalose), lactic acid, and amino acids (Val, Lys, Leu, Ile, Glu) (Supplementary Fig. S2 and Table S1). All NMR identifications were verified using NMR by spiking the exudates with known pure compounds. Some additional compounds were identified by manual analysis of NMR datasets and spiking experiments with known compounds.

GC-MS profiling (Roessner et al., 2000; Wagner et al., 2003; Fiehn et al., 2000) identified 29 compounds in young adult exudates, including the 8 identified by COLMAR. These water-soluble *C. elegans* exudates include several sugars, organic acids, and amino acids (Table 1). The NMR and GC-MS analyses both demonstrate that several free amino acids are in *C. elegans* exudates. As noted above, the NMR spectra also showed that some amino acid concentrations—most obviously the methyl resonances of Val, Leu, and Ile—significantly change with development (Fig. 1). We therefore quantified amino acid concentrations by HPLC. Principal component analysis (PCA) was performed on the amino acid profile data to assess overall experimental quality and variation between each developmental stage. PCA revealed that the three highest-ranking components accounted for

90.1% of the total variance within the dataset. The first two components (81% of the total variance) were enough to distinguish the individual life stages (Fig. 2A), demonstrating that development is a significant factor in the variation observed in *C. elegans* exudates. Furthermore, the variance from experiment to experiment was small in L1 and L2, but increased as the development progressed. This may be due in part to small differences in initial synchronization of worms that propagate with time.

The concentration of every amino acid in *C. elegans* exudates changed with development (Fig. 2B). Of all the developmental stages, L1 contained the fewest amino acids, with only 7 (Ala, Glu, Asp, Ile, Leu, Lys, Phe) detected and present at low concentrations. Every amino acid increased in concentration from L1 to L2 and from L2 to L3. For most developmental stages, Ala and Glu were the most abundant amino acids, with Ala at an approximately 10-fold average higher concentration than most of the others. Several amino acids (Ile, Leu, Thr, Pro, Phe, Met, Tyr, and Ala) have a profile that is highest at the L3 and young adult stages with a slight decrease at L4. With the exception of Asp, Lys, and ornithine (Orn), all amino acid concentrations decreased from young adult to adult stages. The concentration of Orn steadily increased through adult (Fig 2B).

To rule out the possibility that the identified molecules originated from the bacterial food source, we repeated the HPLC and GC-MS experiments on a control preparation that contained bacteria without worms; this sample was prepared identically to the worm exudate preparations, and we did not observe any amino acids or other compounds in Table 1. Additionally, the variation in metabolite profiles between different *C. elegans* life stages also suggests that bacteria, a constant in the experiments, are not the direct source of the observed metabolites. In summary, *C. elegans* exudates contain at least 36 amino acids, sugars and organic acids, and the concentration of many of these compounds vary with development.

Bacteria Chemotax towards *C. elegans* Exudates

Several species of bacteria, including *E. coli*, chemotax in response to various small molecule cues (Adler, 1966; Mesibov and Adler, 1972; Adler et al., 1973; Hedblom and Adler, 1983; Tso and Adler, 1974; Bacilio-Jiménez et al., 2003; Kumar et al., 2007; Lugtenberg et al., 1999; Singh and Arora, 2001; van der Drift et al., 1975). For example, *E. coli* is attracted to Asp, Ser, Glu, Ala, Asn, Gly, Cys, Met, Thr, and trehalose (Adler et al., 1973; Hedblom and Adler, 1983; Mesibov and Adler, 1972) and repelled by Leu, Ile, Val, Trp, Phe, Gln, and His (Tso and Adler, 1974). *C. elegans* lives in a very complex soil environment with both beneficial and pathogenic microbial communities, and the chemical composition of *C. elegans* exudates suggests that the worms might attract bacteria. To test this hypothesis, exudates from young adult animals were qualitatively tested for bacterial chemotaxis using the agarose-in-plug bridge method (Yu and Alam, 1997). We tested three bacterial species: *Pseudomonas aeruginosa* (PAO1), a bacterial species pathogenic to *C. elegans* (Aballay and Ausubel, 2002; Darby et al., 1999), *Pseudomonas putida* (KT2440), a plant growth promoting rhizobacterium (Molina et al., 2000; Ramos-Gonzalez et al., 2005), and *E. coli* (OP50), a widely used food source for *C. elegans* that is defective in motility. *P. aeruginosa* (PAO1) and *P. putida* (KT2440) both exhibited positive chemotaxis (Fig. 3A).

This result is consistent with the presence of Ser, Glu, alpha-keto glutarate, succinate, citrate, and glucose in young adult exudates, as they have all been shown to attract *P. aeruginosa* (Moulton and Montie, 1979). As a negative control, we tested *E. coli* (OP50), because it is widely accepted that this strain of bacteria is defective in motility (P. Sternberg, personal communication); this bacterium showed no response, consistent with our expectations (data not shown). Because beneficial and harmful bacteria were attracted to *C. elegans* exudates, we also tested the exudates for antibacterial activity against *P. aeruginosa* (PAO1). The exudates showed no antibacterial activity in our assays (data not shown). This is not surprising because previous studies documented higher bacterial activity and growth when *Pseudomonas fluorescens* and *Bacillus subtilis* were co-cultured with *C. elegans* (Huixin et al., 2001).

***C. elegans* Exudates Inhibit Bacterial Quorum Sensing (QS)**

We tested worm exudates for QS inhibition (QSI) activity, which would attenuate the bacterial pathogenicity, but not the growth and development (Bjarnsholt and Givskov, 2007; Dong et al., 2007). All stages of *C. elegans* exudates were tested, and no activity was observed in larval stages (L1-L4). However, exudates from both young adult and adult animals showed QSI activity, as indicated by the presence of the bacterial growth around the well (Fig. 3B). Although the assay used in Fig. 3B is qualitative, the bacterial zone of growth (indicated by the blue color) is larger in exudates from young adults than adults. This suggests that *C. elegans* QSI activity is developmentally regulated.

We then tested *C. elegans* exudates for their ability to interfere with specific pathways in the production of virulence factors in *P. aeruginosa* QS. The exudates did not affect the production of elastase and protease, but induced a small amount of pyocyanin production (Supplementary Fig. S1) (Pearson et al., 1997; Fothergill et al., 2007). This small increase could be due to either an increase in release of pyocyanin, increase in biosynthesis, or both. We were unable to detect the known and commercially available QS inhibitors (patulin and penicillic acid) in *C. elegans* exudates (data not shown); these compounds have been shown to inhibit the *P. aeruginosa* QS system (Rasmussen and Givskov, 2006). These results suggest that the *luxR* QSI activity of *C. elegans* exudates observed in the QSIS1 reporter system (Fig 3B) does not significantly affect the *P. aeruginosa* QS system.

Chemical Fractionation of Bacterial Chemotaxis and QSI Activities

To our knowledge, the data in Fig. 3 are the first demonstration of either bacterial chemotaxis or QSI activity of *C. elegans* exudates. Based on extensive studies of bacterial chemotaxis, most of that activity could be explained by several of the polar compounds in Table 1. In order to demonstrate that chemotaxis and QSI are caused by different chemicals, we fractionated young adult *C. elegans* exudates with a C18 solid phase extraction column (Srinivasan et al., 2008) and tested the activities of flow through as well as the 50% and 90% methanol (MeOH) fractions. The flow through contains polar compounds like many amino acids, organic acids, and sugars, which are known bacterial attractants, while the 50 and 90% MeOH fractions contains more hydrophobic compounds. Fig. 4A shows that both *P. aeruginosa* (PAO1) and *P. putida* (KT2440) chemotax robustly to the flow through, less to

the 50% MeOH, and very little to the 90% MeOH fractions. This is consistent with the literature on bacterial chemotaxis.

To test exudate fractions for QSI activity, we used a reporter system that could be more easily quantified and adapted to high-throughput testing than the assay used in Fig. 3B. In the assay shown in Figs. 4B, 4C, and 4D, fluorescence from the green fluorescent protein (GFP) was monitored as a function of time in a 96 well plate format. If no QSI inhibitor is present, the GFP fluorescence is maximal, and QSI inhibitors will reduce the intensity (Andersen et al., 2001). The QSI data shown in Fig. 4 were normalized to the overall cell growth monitored by the optical density at 450 nm to account for any general effects that the tested compounds have on *E. coli* cell growth. Fig. 4B shows a complete time course that starts with a lag phase to about 40 min., is linear in GFP fluorescence from 40 to about 160 min., and remains stable after 160 min. Figures 4C and 4D are linear fits from 40 to 160 min. of control (4C) and *C. elegans* flow through and 90% MeOH (4D) samples. The linear fitting parameters and correlation coefficients (R^2) and the slopes for all samples assayed in Fig. 4B are shown in Table 2, and P value comparisons of QSI activity between each sample are shown in Table 3.

The negative control (water) and *C. elegans* flow through fractions were similar ($P = 0.5$; Table 3), demonstrating that the flow through has no QSI activity. The positive control (12.5 μ M 4-nitropyridine N-oxide) and 90% MeOH fractions were also similar ($P = 0.3$), and the 90 % MeOH fraction was distinct from both the negative control and flow through ($P < 0.001$). These results demonstrate that the QSI activity results from a non-polar compound(s), in contrast to the polar flow through compounds that are responsible for bacterial chemotaxis. The 50% MeOH fraction has intermediate QSI activity (Tables 2 and 3). We also compared exudates before fractionation and reconstituted after fractionation to verify that the C18 column did not degrade or irreversibly bind to the compound responsible for the QSI activity, and these both are significantly different from the negative control ($P < 0.001$) (Tables 2 and 3).

DISCUSSION

We have shown that *C. elegans* releases a variety of sugars, organic acids, and amino acids into its environment (Table 1) and that the concentrations of the amino acids are dependent upon development. Many of these compounds are also found in the exudates of other organisms including bacteria (Park et al., 2003; Zahradníková et al., 2007), fungi (Singh and Arora, 2001), and several species of plants (Bacilio-Jiménez et al., 2003; Kumar et al., 2007; Lugtenberg et al., 1999; Kamilova et al., 2006). Additionally, many plant root exudates, and fungal or bacterial exudates attract bacteria (Bacilio-Jiménez et al., 2003; Kumar et al., 2007; Lugtenberg et al., 1999; Singh and Arora, 2001; Park et al., 2003). Given their chemical composition, it was not surprising that *C. elegans* exudates examined in this work attracted bacteria. Previous experiments have shown that *C. elegans* enhance the growth of *Pseudomonas fluorescens* and *Bacillus subtilis* (Huixin et al., 2001). This suggests a hypothesis that *C. elegans* plays an active role in soil chemical ecology and may have evolved chemical systems for attracting and promoting growth of its own food source. Relatively little is known about the range of bacterial species that *C. elegans* eats in its

native environments (Shtonda and Avery, 2006; Avery and Shtonda, 2003), so the hypothesis of “bacterial farming” by *C. elegans* remains to be tested. It should be noted that we only investigated water-soluble compounds that are released from the worms, and other compounds may interact with the hydrophobic cuticle or may be volatile.

If *C. elegans* non-specifically attracts bacteria, it is possible that pathogenic species have evolved to take advantage of this otherwise beneficial survival mechanism. Depending on the bacterial composition in a particular environment, one might predict that *C. elegans* would therefore also need to employ some sort of defense against pathogens or colonize soil patches that are devoid of pathogens. However, we were unable to detect any *C. elegans* antibacterial activity against the pathogenic bacteria *P. aeruginosa*, despite the fact that this same pathogen was attracted to *C. elegans* exudates. Since *P. aeruginosa* is resistant to many antibiotics (Stover et al., 2000), we cannot completely rule out the possibility that *C. elegans* produces antibacterial compounds, or that antibacterial compounds might be present in the intestine or on the cuticle of the nematode rather than released. Even though the data and literature are best explained by the absence of antibacterial compounds in *C. elegans* (Huixin et al., 2001), a result that is ecologically consistent with a bacterivorous nematode, this does not eliminate the possibility that *C. elegans* may have an inducible system to produce antibacterial activity.

Many pathogenic bacteria, including *P. aeruginosa*, regulate virulence through quorum sensing (Dong et al., 2007; Bjarnsholt and Givskov, 2007), and *P. aeruginosa* (PAO1) is lethal to *C. elegans* via a toxin produced through the *LasR* QS system (Darby et al., 1999). Many species of bacteria, fungi, and plants employ a variety of mechanisms to inhibit bacterial QS, including enzymatic degradation of QS signaling molecules and direct inhibition (Dong et al., 2007; Bais et al., 2005; Walker et al., 2004). We showed that *C. elegans* exudates inhibited a *LuxR* QS system that was derived from *Vibrio fischeri*. However, the worm exudates did not affect common biomarkers of QS in *P. aeruginosa* (PAO1), the production of elastase and protease, and they had only a small influence on pyocyanin production (Supplementary Fig. S1). The *C. elegans* QSI activity appears developmentally regulated with maximal activity at the young adult stage. Perhaps *C. elegans* has evolved a mechanism by which it can avoid infection by certain pathogenic species of bacteria without employing antibiotics. These results are consistent with the observations that *C. elegans* exudates attract several bacterial species, as the QSI activity would provide the nematodes with a chemical defense without killing their food source.

In summary, *C. elegans* releases many common small molecules that can modulate its environment, and many of these compounds are developmentally regulated. These compounds may play important roles in the ecology of *C. elegans* in its natural habitat. Our data suggest that these molecules can provide a means for the worms to interact with bacterial food sources and potentially to defend against bacterial pathogens. It remains an open question whether the bacterial chemoattractants or quorum sensing inhibitor(s) are evolutionary adaptations or are byproducts of another process, such as metabolism. The identification of the QSI will help address this question and may open up new areas of research into the chemical ecology of nematodes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

NMR spectra of *C. elegans* exudates as a function of development. One-dimensional ^1H NMR spectra containing 54,000 WE from different *C. elegans* developmental stages were collected from three independent preparations of each developmental stage. The data shown are from one preparation and are representative of the others. Three regions of the spectra are shown, demonstrating a large amount of variation in chemical composition as a function of development. The annotations at the top are on the young adult exudates. The compounds were identified using either the semi-automated COLMAR analysis or manual analysis and spiking experiments, as described in the text.

Fig. 2.

Quantitative analysis of amino acids released by *C. elegans* at different developmental stages. Three independent preparations of exudates from each developmental stage were analyzed by HPLC. (A) Principle component analysis of all developmental stages. Each circle represents an independent experiment. The L1 data include three datasets that had very small variance among the three experiments. Components 1 and 2 account for 81% of the variation in the HPLC dataset. (B) Plots of amino acid concentrations (picomoles per worm equivalent) as a function of development. Note that the vertical concentration scale in each of the panels in B is different. Each point is the average and standard deviation of the three independent sample preparations and HPLC measurements. The amino acids are grouped according to overall shape of their concentration profile. The 7 in the top panel (Ile, Leu, Thr, Pro, Phe, Met, and Tyr) all have a peak at L3, a slight dip at L4, a slight increase at young adult (YA) and a drop at adult (A) stages. The group of 4 amino acids in the middle panel (Val, Asp, Lys, and Arg) are more variable, and the 2 amino acids at in the bottom panel (Ala and Glu) are produced in significantly higher concentrations.

Fig. 3. Qualitative assays for bacterial chemotaxis and quorum sensing inhibition activity of *C. elegans* exudates. (A) Chemotaxis of *P. putida* (KT2440) and *P. aeruginosa* (PAO1). The arrow marks indicate the margin of the agarose plug. Dark spots near the margin of agarose plug are the bacterial cells. The bacterial cell density around an agarose plug that contained the exudates of interest was observed with a 40X phase-contrast objective (GX microscope) 30 minutes after introduction of a bacterial suspension. One plane of focus adjacent to the plug was photographed with a CCD camera on the microscope. LB media is a positive control and chemotaxis buffer is a negative control. (B) The QSI reporter system (Rasmussen et al., 2005a) was employed for these qualitative assays. Blue color around the well indicates QSI activity. The top (sterile water) and bottom (*E. coli*) panels are negative controls, and chamomile extract is a positive control. *C. elegans* young adult and adult exudates showed positive QSI activity. For both the chemotaxis and QSI assays, three independent preparations of worm exudates were produced, and three assays were conducted on each preparation, for a total of 9 assays. The figures are representative examples of one of these assays for each activity.

Figure 4.

Fractionation of bacterial chemotaxis and QSI activity. Young adult *C. elegans* exudates were separated into flow through, 50% methanol (MeOH), and 90% MeOH fractions by C18 solid phase extraction. (A) Chemotaxis of *P. aeruginosa* (PAO1) and *P. putida* (KT2440), as described in Figure 3. The chemotaxis assay was repeated 3 times on 2 different sample preparations for a total of 6 replicates. Each of the assays showed the greatest bacterial chemotaxis towards the hydrophilic flow through fractions and very little chemotaxis to the most hydrophobic 90% MeOH fractions. The figures in A are a representative dataset. (B, C, and D) Quantitative QSI assay of young adult *C. elegans* C18 fractions (Andersen et al., 2001). B is a complete 220 minute time course with the GFP fluorescence normalized to the bacterial optical density monitored at 450 nm. The increase in fluorescence is reduced by compounds with QSI activity, as seen by comparing the blue sterile water negative control with the pink 12.5 μ M 4-nitropyridine N-oxide (4NPO) positive control. The data shown in B represent 3 replicate assays on 1 sample preparation. C and D show the data and linear fits from 40 to 160 minutes for negative and positive controls (C) and the flow through and 90% MeOH fractions (D). The negative and positive controls in C were from 3 replicates, and the exudate fractions in D were from 3 replicate measurements of 3 independent samples (total of 9 measurements). The slopes, intercepts, and fitting statistics for the data shown in C and D as well as all the other conditions from B are in Table 2. We also compared the differences between each sample set using the model comparison technique under the linear model framework, and the statistical p values are shown in Table 3. The *C. elegans* flow through fraction had no QSI activity when compared with negative control ($p=0.5$), and there is no significant difference between the 90%MeOH fraction and positive control,

showing that the hydrophobic fraction has QSI activity. The statistical analyses were performed using R2.8.0.

Table 1**CHEMICAL COMPOSITION OF *C. ELEGANS* YOUNG ADULT EXUDATES^a**

Sugars	Amino acids	Other compounds
Fructose [#]	Glycine [#]	2-Ketoglutaric acid [#]
Glucose ^{##}	L-Alanine ^{*\$#}	Allontoin [#]
Glucose-6-P [#]	L-Glutamic acid ^{*\$#}	Anthranilic acid [#]
Trehalose ^{*#}	L-Isoleucine ^{*\$#}	Betaine [*]
	L-Leucine ^{*\$#}	Citric acid [#]
	L-Lysine ^{*\$#}	Erythronic acid [#]
Amino acids	L-Phenylalanine ^{*\$#}	Fumaric acid [#]
2-Aminoadipic acid ^{*\$#}	L-Serine [#]	Glutaric acid [#]
Beta-Alanine ^{*\$#}	L-Threonine ^{\$#}	Lactic acid ^{*#}
N-Acetylglutamic acid ^{\$}	L-Tyrosine ^{\$#}	Malic acid [#]
Ornithine ^{**\$}	L-Valine ^{*\$#}	myo-Inositol [#]
Proline ^{\$}	Aspartate ^{\$}	Threonic acid [#]
Methionine ^{\$}	Arginine ^{\$}	Urea [#]

^aMetabolites were identified by combined approaches of NMR, GC-MS, and HPLC.

* Identified and verified by NMR,

** Identified but not verified by NMR,

[#] identified by GC-MS,

^{\$} identified by HPLC.

Three independent experiments were done starting from worm growth for GC-MS, HPLC and only two of those used for NMR. We also had 4 additional experiments run on HPLC. As a control, *E. coli* (HB101) was treated the same way as the worms to determine any contamination from food source. Identification of glucose-6-P is with 80% confidence.

Table 2

PARAMETERS FROM LINEAR REGRESSION ANALYSIS OF QSI DATA FROM FIGURES 4C AND 4D.^a

Sample	Slope (STD)	Intercept (STD)	R
Flow Through	678.7 (20.1)	-21593.9 (3170.1)	0.95
Negative Control	729.0 (32.9)	-26314.2 (3540.0)	0.96
Positive Control	567.7 (33.6)	-24444.3 (3615.1)	0.94
Prefractionation	579.3 (19.7)	-18866.7 (2119.2)	0.98
Reconstituted	553.0 (22.5)	-17641.0 (3352.4)	0.94
50% MeOH	616.4 (20.3)	-22427.2 (3349.5)	0.94
90% MeOH	535.2 (16.4)	-19133.7 (3107.7)	0.95

^a All fits had P values <0.0001.

Table 3

COMPARISON OF THE LINEAR MODELS FOR QSI ACTIVITY FROM THE DIFFERENT SAMPLES SHOWN IN FIGURE 4B.^a

	NC	PC	Pre	Rec	50% MeOH	90% MeOH
Flow Through	5.10E-01	<0.001	<0.001	<0.001	<0.001	<0.001
Negative Control (NC)		<0.001	<0.001	<0.001	<0.001	<0.001
Positive Control (PC)			<0.001	1.27E-02	<0.001	3.17E-01
Prefractionation (Pre)				5.43E-01	6.79E-01	4.56E-03
Reconstituted (Rec)					1.19E-01	3.97E-02
50% MeOH						<0.001

^aThe entries are P values, and numbers greater than 0.05 indicate that the two samples are not statistically different.
The highlighted boxes show that the *C. elegans* flow through has no QSI activity and that the 90% MeOH fraction has similar activity to the 12.5 μM 4NPO positive control.