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Caenorhabditis elegans Senses Bacterial Autoinducers

Elmus Beale,[1] Guigen Li,[3] Man-Wah Tan,[4] and Kendra P. Rumbaugh[2]*

Departments of Cell Biology and Biochemistry[1] and Surgery,[2] Texas Tech University Health Sciences Center, and Department of Biochemistry, Texas Tech University,[3] Lubbock, Texas 79430, and Department of Genetics, Stanford University School of Medicine, Stanford, California 94305[4]

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***Pseudomonas aeruginosa* uses virulence factors controlled by quorum sensing (QS) to kill *Caenorhabditis elegans*. Here we show that *C. elegans* is attracted to the acylated homoserine lactones (AHSLs) that mediate QS in *P. aeruginosa*. Our data also indicate that *C. elegans* can distinguish AHSLs and may use them to mediate aversive or attractive learning.**

Caenorhabditis elegans is a free-living terrestrial nematode that feeds on bacteria in its environment. Although little is known about how *C. elegans* finds food sources, it does possess a sophisticated chemosensory system that enables it to sense and respond to a wide range of chemicals (2). Recent data show that *C. elegans* uses odors produced by bacteria to identify food sources, although no specific odorants have been identified (16). Indeed, the identities of natural products that *C. elegans* senses and responds to remain unknown. In this study we examined whether *C. elegans* could sense the acylated homoserine lactone (AHSL) autoinducers produced by many gram-negative bacteria possessing quorum-sensing (QS) systems. Numerous bacterial functions have been attributed to AHSL signaling, including virulence factor production in *Pseudomonas aeruginosa* (13). Several recent studies suggest the AHSLs not only function in bacterial signaling but also are capable of modulating several signaling pathways in eukaryotic cells, a process we previously termed "interkingdom signaling"(13).

While *Pseudomonas* species can serve as food for *C. elegans*, some species are also pathogens (1). Utilizing standard chemotaxis assays, we compared the attraction of *C. elegans* to AHSL-producing (PAO1) and non-AHSL-producing (PAO1-JP2) (10) strains of *P. aeruginosa*. The PAO1-JP2 strain has deletions in the *lasI* and *rhlI* autoinducer synthase genes and is defective in autoinducer production and the production of QS-controlled exoproducts (10). In these assays *C. elegans* migrates toward one of two bacterial lawns on opposite sides of an agar plate as described previously (2). Briefly, 50-μl volumes of PAO1 and PAO1-JP2 overnight growth were spotted on chemotaxis plates (nematode growth medium lacking cholesterol) 6 cm apart and were grown at 37°C for 24 h. Fifteen minutes prior to the assay, 1 μl of 1 M NaN_3 was applied to the centers of the bacterial lawns to anesthetize any worms that reached the spot during the experiment. Approximately 200 well-fed adult *C. elegans* wild-type strain Bristol N2 worms were rinsed in M9 medium, placed equidistantly between the two bacterial lawns, and allowed to undergo chemotaxis for 1 h. The worms were then counted using a dissecting microscope, and the chemotaxis index (CI) was determined for each plate. The CI is defined as the number of worms within a 2-cm radius of the test spot (in this case the PAO1 lawn) minus the number of worms within a 2-cm radius of a control spot (PAO1-JP2 lawn), divided by the total number of worms on the plate. (Note: the CI range is from −1 to +1, with 0 being neutral, +1 being a perfect attractant, and −1 being a perfect repellant). *C. elegans* worms migrated towards the PAO1 lawn approximately 40% more often than towards the PAO1-JP2 lawn (CI = 0.23 ± 0.08, $P = 0.003$, Student's t test; nine assays). This indicates that *C. elegans* was either attracted to an odorant made by PAO1 but not by PAO1-JP2 or repelled by an odorant made by PAO1-JP2 but not by PAO1. To examine the second possibility, chemotaxis assays were performed in which worms were given the food choices of PAO1 versus OP50 or PAO1-JP2 versus OP50. *Escherichia coli* OP50 is a standard bacterial food source for *C. elegans* and is used to maintain worm strains in the laboratory. We reasoned that if PAO1-JP2 possessed a repellant that PAO1 did not, we would detect a difference in the CIs between the two food choices. That worms preferred OP50 over both PAO1 and PAO-JP2, with identical CIs (CI = 0.32 ± 0.04 and 0.32 ± 0.05, respectively; $n = 5$), suggests that worms are not more repelled by PAO1-JP2 than by PAO1. We thus proceeded with the assumption that PAO1 possesses an attractant that is absent from PAO1-JP2.

Attraction to autoinducers. As there are several bacterial products other than AHSLs that are generated by PAO1 but not PAO1-JP2, we next tested whether *C. elegans* was attracted to pure synthetic AHSLs (synthesized in our laboratory as previously described [4]). Four different AHSLs were tested, including two that are naturally synthesized by *P. aeruginosa* (3O-C_{12}-HSL and C_4-HSL), one synthesized by *Agrobacterium tumefaciens* (3O-C_8-HSL) and C_{12}-HSL, an analog of 3O-C_{12}-HSL, that is significantly less active in bacteria (Fig. 1). For these assays, 10 μM of autoinducer was spotted on a test spot 6 cm away from a control spot. In standard *P. aeruginosa* cultures, 3O-C_{12}-HSL concentrations have been measured in the range of 2 to 10 μM (9, 14). However, concentrations of up to 600 μM have been measured within *P. aeruginosa* biofilms (3). Therefore, we reasoned that concentrations of 10 μM and higher would be expected in *P. aeruginosa* biofilms in nature.

* Corresponding author. Mailing address: Texas Tech University Health Sciences Center, Department of Surgery, 3601 4th Street, Lubbock, TX 79430. Phone: (806) 743-2460, ext. 264. Fax: (806) 743-2370. E-mail: kendra.rumbaugh@ttuhsc.edu.

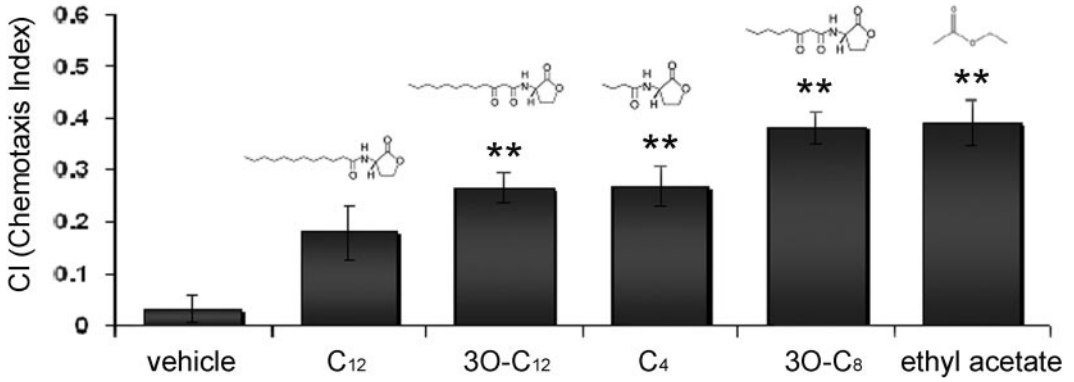

FIG. 1. *C. elegans* worms are attracted to AHSLs. Error bars indicate standard errors of the mean (SEM); $n = 12$; **, $P < 0.0001$ (analysis of variance with Scheffe's post hoc test).

Autoinducers were solubilized in 30 μl of 95% ethanol, which is a known neutral solvent for *C. elegans* (2). Thirty microliters of 95% ethanol was also placed on control spots. NaN_3 was placed on test and control spots 15 min before the assay began. Positive control plates had 5 μl of ethyl acetate (a known strong attractant) on the test spots. Negative control (or vehicle) plates had 95% ethanol on both the test and the control spots. Chemotaxis towards 3O-C_{12}-HSL, 3O-C_8-HSL, and C_4-HSL was significantly greater than that towards vehicle alone, with the CI for each approaching that for ethyl acetate, a potent attractant for *C. elegans* ($P < 0.0001$, analysis of variance with Scheffe's post hoc test; 12 assays) (Fig. 1). The CI for the analog C_{12}-HSL, although elevated, was not significantly greater than that for the vehicle ($P = 0.06$, Student's t test; 12 assays).

Autoinducer-mediated aversive learning. The ability of *P. aeruginosa* to kill *C. elegans* is under QS control (7, 15). Recent data show that after a short exposure to *P. aeruginosa*, *C. elegans* learns to avoid it when subsequently exposed (16). This aversive learning was odor mediated (16). We thus examined whether *C. elegans* could learn to avoid *P. aeruginosa* autoinducers after exposure to a pathogenic *P. aeruginosa* strain. *P. aeruginosa* strain PAO1 kills *C. elegans* via a QS-controlled cyanide (6). Lethal cyanide paralysis by PAO1 begins by 1.5 h after exposure. By 2 h, 30 to 40% of worms are paralyzed, and by 3 h, 100% are paralyzed (6). For our experiments, *C. elegans* worms were placed on lawns of PAO1 or PAO1-JP2 for 1 h. They were then removed prior to paralysis and placed on 3O-C_{12}-HSL chemotaxis plates as described above (Fig. 2A). Strikingly, and in comparison to naïve worms, worms preexposed to PAO1 were repelled by 3O-C_{12}-HSL (CI = -0.24 ± 0.04, $P < 0.001$, Student's t test; 10 assays). However, worms preexposed to the QS mutant PAO1-JP2 were still attracted to 3O-C_{12}-HSL (CI = 0.16 ± 0.06; nine assays).

C. elegans can learn to avoid tastes, odors, and temperatures by positive and/or negative conditioning (11), and this memory can be either short-term (minutes) or long-term (hours to days) (12). We investigated how long avoidance of autoinducer lasted after exposure to PAO1. In these experiments, worms were fed on lawns of PAO1 for 1 h as described above. Worms were then deprived of food for 4 or 8 h prior to being placed on 3O-C_{12}-HSL chemotaxis plates as described above. After 4 h of fasting, worms still avoided 3O-C_{12}-HSL similarly to worms that had not been deprived of food (CI = -0.24 ± 0.04, $P = 0.7$, Student's t test; 11 assays) (Fig. 2B). After 8 h of fasting, although the avoidance behavior was diminished compared to that after no fasting, it was still detectable (CI =

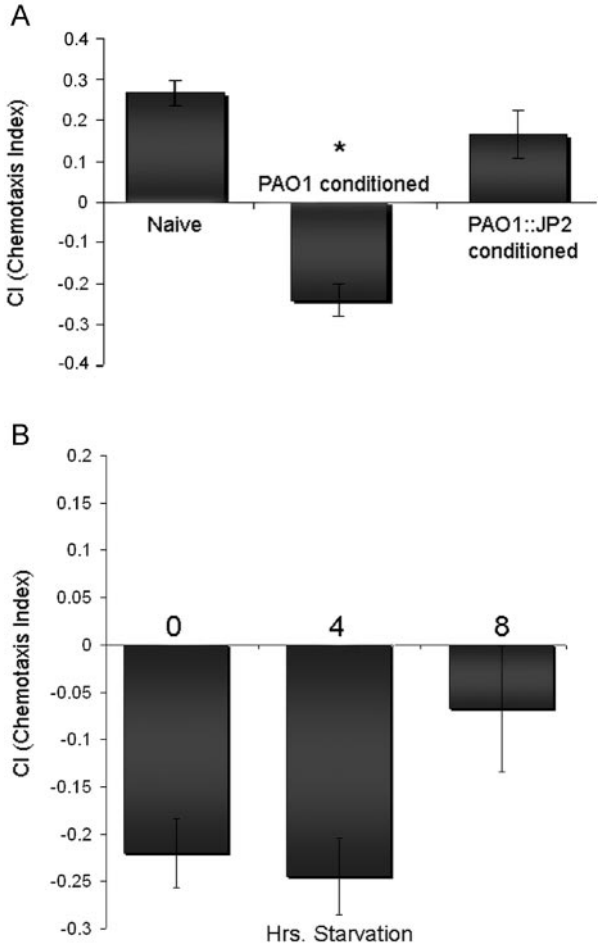

FIG. 2. (A). *C. elegans* worms are repelled by 3O-C_{12}-HSL after exposure to PAO1 but not PAO1-JP2. (B). Autoinducer avoidance after PAO1 exposure is long lasting. Error bars indicate SEM; $n = 10$; *, $P < 0.001$ (Student's t test).

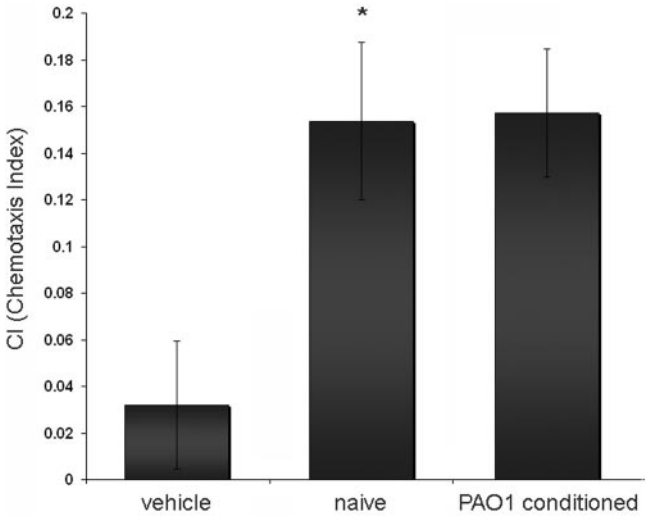

FIG. 3. *mod-1* mutant worms are attracted to 3O-C_{12}-HSL but do not display aversive learning after PAO1 exposure. Error bars indicate SEM; $n = 10$; *, $P = 0.02$ (Student's t test).

-0.07 ± 0.05, $P = 0.06$, Student's t test; 10 assays). Fasting alone did not result in 3O-C_{12}-HSL avoidance (data not shown). Taken together, these data show that *C. elegans* can learn to avoid autoinducers and that this avoidance appears to be relatively long lasting.

Autoinducer-mediated positive conditioning. As *C. elegans* can make a negative association with autoinducers following exposure to pathogenic *P. aeruginosa*, we wanted to determine if a positive association could also be made. For these experiments, worms were allowed to feed on lawns of OP50 to which either autoinducer or vehicle had been added. As attractive learning has been shown to require longer conditioning times (16), Bristol N2 worms were allowed to feed for 4 h, were fasted for 2 h, and then were removed and placed on 3O-C_{12}-HSL chemotaxis plates as described above. Worms that fed on OP50 plus autoinducer were subsequently more attracted to 3O-C_{12}-HSL than those that fed on OP50 plus vehicle (CI = 0.22 ± 0.03 and 0.13 ± 0.02, respectively, $P = 0.045$, Student's t test; 10 assays). Therefore, *C. elegans* displays both attractive and aversive learning to autoinducers.

Olfactory learning. Serotonin-mediated signal transduction has been implicated in olfactory learning in *C. elegans* (8, 16). MOD-1 is a serotonin-gated chloride channel expressed in sensory neurons. Zhang et al. recently demonstrated that *C. elegans mod-1* mutant worms were defective in aversive olfactory learning after exposure to pathogenic bacteria (16). As aversive learning requires serotonin from ADF neurons and the MOD-1 serotonin receptor, we examined whether naïve or PAO1-conditioned *mod-1* mutant worms were attracted to *P. aeruginosa* 3O-C_{12}-HSL by utilizing chemotaxis assays as described above (Fig. 3). Naïve and PAO1-conditioned *mod-1* mutant worms were attracted to 3O-C_{12}-HSL, indicating that defects in serotonin-mediated signaling disrupt aversive olfactory learning to autoinducer. In addition, the Toll-interleukin 1 protein is also involved in odorant receptor expression in *C. elegans* AWB neurons (5). However, we determined that attraction to autoinducers is not defective in *tol-1* mutants (data not shown).

Concluding remarks. In summary, *C. elegans* encounters many strains of bacteria in its natural soil environment. The ability to find good food sources over potential pathogens is a significant advantage. *C. elegans* uses a sophisticated chemosensory system to identify food and olfactory learning as a mechanism to avoid pathogens. This conditioning behavior is analogous to mammalian taste aversion. While there are many odorants produced by bacteria that *C. elegans* may detect and/or learn to avoid, we show here that one type of bacterial chemical, AHSL autoinducers, can mediate aversive and attractive learning.

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REFERENCES

1. **Alegado, R. A., M. C. Campbell, W. C. Chen, S. S. Slutz, and M. W. Tan.** 2003. Characterization of mediators of microbial virulence and innate immunity using the *Caenorhabditis elegans* host-pathogen model. Cell. Microbiol. **5:**435–444.
2. **Bargmann, C. I., E. Hartwieg, and H. R. Horvitz.** 1993. Odorant-selective genes and neurons mediate olfaction in *C. elegans*. Cell **74:**515–527.
3. **Charlton, T. S., R. de Nys, A. Netting, N. Kumar, M. Hentzer, M. Givskov, and S. Kjelleberg.** 2000. A novel and sensitive method for the quantification of N-3-oxoacyl homoserine lactones using gas chromatography-mass spectrometry: application to a model bacterial biofilm. Environ. Microbiol. **2:**530–541.
4. **Chhabra, S. R., C. Harty, D. S. Hooi, M. Daykin, P. Williams, G. Telford, D. I. Pritchard, and B. W. Bycroft.** 2003. Synthetic analogues of the bacterial signal (quorum sensing) molecule N-(3-oxododecanoyl)-L-homoserine lactone as immune modulators. J. Med. Chem. **46:**97–104.
5. **Chuang, C. F., and C. I. Bargmann.** 2005. A Toll-interleukin 1 repeat protein at the synapse specifies asymmetric odorant receptor expression via ASK1 MAPKKK signaling. Genes Dev. **19:**270–281.
6. **Darby, C., C. L. Cosma, J. H. Thomas, and C. Manoil.** 1999. Lethal paralysis of *Caenorhabditis elegans* by *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **96:**15202–15207.
7. **Gallagher, L. A., and C. Manoil.** 2001. *Pseudomonas aeruginosa* PAO1 kills *Caenorhabditis elegans* by cyanide poisoning. J. Bacteriol. **183:**6207–6214.
8. **Nuttley, W. M., K. P. Atkinson-Leadbeater, and D. Van Der Kooy.** 2002. Serotonin mediates food-odor associative learning in the nematode *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. USA **99:**12449–12454.
9. **Pearson, J. P., K. M. Gray, L. Passador, K. D. Tucker, A. Eberhard, B. H. Iglewski, and E. P. Greenberg.** 1994. Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. Proc. Natl. Acad. Sci. USA **91:**197–201.
10. **Pearson, J. P., E. C. Pesci, and B. H. Iglewski.** 1997. Roles of *Pseudomonas aeruginosa las* and *rhl* quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. J. Bacteriol. **179:**5756–5767.
11. **Rankin, C. H.** 2004. Invertebrate learning: what can't a worm learn? Curr. Biol. **14:**R617–R618.
12. **Rose, J. K., and C. H. Rankin.** 2001. Analyses of habituation in *Caenorhabditis elegans*. Learn. Mem. **8:**63–69.
13. **Shiner, E. K., K. P. Rumbaugh, and S. C. Williams.** 2005. Interkingdom signaling: deciphering the language of acyl homoserine lactones. FEMS Microbiol. Rev. **29:**935–947.
14. **Smith, R. S., E. R. Fedyk, T. A. Springer, N. Mukaida, B. H. Iglewski, and R. P. Phipps.** 2001. IL-8 production in human lung fibroblasts and epithelial cells activated by the *Pseudomonas* autoinducer N-3-oxododecanoyl homoserine lactone is transcriptionally regulated by NF-kappa B and activator protein-2. J. Immunol. **167:**366–374.
15. **Tan, M. W., L. G. Rahme, J. A. Sternberg, R. G. Tompkins, and F. M. Ausubel.** 1999. *Pseudomonas aeruginosa* killing of *Caenorhabditis elegans* used to identify *P. aeruginosa* virulence factors. Proc. Natl. Acad. Sci. USA **96:**2408–2413.
16. **Zhang, Y., H. Lu, and C. I. Bargmann.** 2005. Pathogenic bacteria induce aversive olfactory learning in *Caenorhabditis elegans*. Nature **438:**179–184.