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A natural odor attraction between lactic acid bacteria and the nematode *Caenorhabditis elegans*

Jae Im Choi, Kyoung-hye Yoon, Saraswathi Subbammal Kalichamy, Sung-Sik Yoon and Jin Il Lee

Division of Biological Science and Technology, College of Science and Technology, Yonsei University, Wonju, Korea

Animal predators can track prey using their keen sense of smell. The bacteriovorous nematode *Caenorhabditis elegans* employs sensitive olfactory sensory neurons that express vertebrate-like odor receptors to locate bacteria. *C. elegans* displays odor-related behaviors such as attraction, aversion and adaptation, but the ecological significance of these behaviors is not known. Using a combination of food microbiology and genetics, we elucidate a possible predator–prey relationship between *C. elegans* and lactic acid bacteria (LAB) in rotting citrus fruit. LAB produces the volatile odor diacetyl as an oxidized by-product of fermentation in the presence of citrate. We show that *C. elegans* is attracted to LAB when grown on citrate media or *Citrus medica L*, commonly known as yuzu, a citrus fruit native to East Asia, and this attraction is mediated by the diacetyl odor receptor, ODR-10. We isolated a wild LAB strain and a wild *C. elegans*-related nematode from rotten yuzu, and demonstrate that the wild nematode was attracted to the diacetyl produced by LAB. These results not only identify an ecological function for a *C. elegans* olfactory behavior, but contribute to the growing understanding of ecological relationships between the microbial and metazoan worlds.

Introduction

For decades, field biologists have elegantly described behaviors that arise due to ecological pressures. However, with the onset of the genome era, scientists are beginning to look at ecology through a molecular lens, focusing on the role of genes and proteins in producing ecologically relevant behaviors. This has led to laboratory-based experiments that have begun to elucidate molecular mechanisms of behavioral ecology. For instance, specific receptors for both pheromones and odors were shown to be important for mice to identify and evade predators such as cats, rats and mountain lions (Papes *et al.,* 2010; Ferrero *et al.,* 2011).

Caenorhabditis elegans, a free-living soil nematode, has been used in the laboratory as a genetic model organism for decades. *C. elegans* displays many robust behaviors related to sex, temperature and food searching (Hedgecock and Russell, 1975; Loer and Kenyon, 1993; Liu and Sternberg, 1995; Hills *et al.,* 2004; Gray *et al.,* 2005). In addition, this

nematode can sense dozens of odors through several olfactory sensory neurons (Bargmann *et al.,* 1993), and shows attraction to, aversion and memory of many of these odors (Ward, 1973; Dusenbery, 1975; Bargmann and Horvitz, 1991; Colbert and Bargmann, 1995). Odors are detected through G-protein coupled odor receptors (GPCRs) similar to receptors in mammals (Buck and Axel, 1991; Bargmann *et al.,* 1993). For instance, the odor diacetyl is sensed by the ODR-10 receptor (Sengupta *et al.,* 1996), one of hundreds of G-protein coupled odor receptor-encoding genes that are scattered throughout the *C. elegans* genome (Robertson, 1998).

Although a plethora of studies have shown the neurons, circuitry and molecules responsible for odor behaviors, the ecological significance of these behaviors in nature are not known. In the laboratory, *C. elegans* is maintained on agar plates and feed on *Escherichia coli*, a bacteria normally found in the intestine of animals, and rarely encountered in nature. In the wild, *C. elegans* can be found in the soil throughout the world, but recent studies have shown that they are gathered in large numbers in rotten fruit and vegetation (Barriere and Felix, 2005; Felix and Braendle, 2010). The exact reason for this is not fully known; however, rotten fruit and vegetation are a rich source of bacteria for worms to feed on. Rhabditid nematodes like *C. elegans* are considered enrichment-type nematodes

Correspondence: S-S Yoon or J Il Lee, Division of Biological Science and Technology, College of Science and Technology, Yonsei University, 1 Yonseidae-gil, Wonju, Gangwondo 220-710, Korea.
E-mail: sungsik@yonsei.ac.kr or jinillee@yonsei.ac.kr

(Bongers, 1990; Darby *et al.,* 2007). They normally exist in a semi-hibernating dauer larval stage in nature (Barriere and Felix, 2005), but when they come upon a rich source of bacteria, the dauers will quickly grow to sexually mature adults, rapidly reproduce in very large numbers, and consume much of the bacteria present (Bongers, 1990). Since fruits are a rich source of sugar and nutrients for bacteria, large bacterial bonanzas of many different species grow and ferment the fruit over time. If *C. elegans* can sense and track rotting fruit, they will likely find bacterial bonanzas blooming throughout, allowing the nematodes to reproduce and flourish.

Lactic acid bacteria (LAB) are a clade of gram-positive bacteria that produce lactic acid as an end product of carbohydrate fermentation. They are found in fermenting vegetative matter and milk, and are major contributors to the production and flavoring of fermented foods such as yogurts and cheeses (Marshall, 1987; Smit *et al.,* 2005). LAB also are found in the human gut, and are associated with positive effects to health (Guarner and Malagelada, 2003; Hempel *et al.,* 2012). Hence, many LAB are also considered to be probiotic. LAB can survive well in low-pH environments such as fruit, which is a tremendous source of sugar and nutrients to growing bacterial populations (Trias *et al.,* 2008). One of the fermentative strategies that LAB has at its disposal when grown on fruit is to utilize citrate together with sugar (Hugenholtz, 1993). A by-product of this fermentative pathway is the production of the volatile chemical diacetyl (butane-2,3-dione) (Hammer, 1920; Michaelian *et al.,* 1933, 1938), which emits a buttery odor that is often used commercially in popcorn.

A few studies have explored the relationship between *C. elegans* and LAB in the laboratory. Exposure to LAB can induce the expression of genes involved in innate immunity providing protection for *C. elegans* particularly towards gram-positive bacterial infection (Ikeda *et al.,* 2007; Kim and Mylonakis, 2012). However, little is understood about the ecological relationship between nematodes and LAB in nature. As *C. elegans* can be found in rotting fruit and has a strong attraction for diacetyl (Bargmann *et al.,* 1993), one possibility is that production of diacetyl from LAB growing on fruit may be one of the cues that nematodes use to find bacterial bonanzas. In this study, we show that LAB grown on citrate media or citrus fruit media produces diacetyl, and *C. elegans* is attracted to diacetyl produced by LAB *via* the ODR-10 diacetyl receptor. We tested LAB and rhabditid nematodes isolated from citrus fruit, and showed that these nematodes are naturally attracted to diacetyl-producing LAB. We speculate that the by-product diacetyl produced from LAB grown on citrate is an environmentally relevant cue that allows nematodes to track bacterial bonanzas located in citrus fruit.

Materials and methods

Strains and maintenance
All *C. elegans* strains were maintained at 20 °C on nematode growth medium (NGM) seeded with OP50 strain *E. coli* (Lewis and Fleming, 1995). Bristol N2 wild type and CX32 *odr-10(ky32)* were obtained from the Caenorhabditis Genetics Center. *Lactobacillus paracasei* strain YSM0385 was isolated from yogurt in the Yoon lab (Song *et al.,* 2013). LAB was grown at 37 °C on Difco Lactobacillus MRS Broth (BD, Sparks, MD, USA, Cat# 288130) before inoculating on to different agar media plates used in the study.

Collection and identification of wild nematode
Collection of soil+rotten fruit samples occurred at the following locations: Se Wook Farms (Wonju), Chiaksan Peach Farms (Wonju), Ja So Dahm Farms (Goheung) and a private farm in Chungju. Wild nematode strains were isolated and identified following published methods (Barrière and Félix, 2014). Briefly, a small portion of the samples were placed on the edge of NGM plates with an *E. coli* OP50 lawn in the middle. Individual or a group of worms were picked into separated seeded NGM plates. Worms were first categorized by morphology, and several worms from each category were sequenced. Sequences from 18S rDNA and ITS2 region were PCR amplified and compared against known sequences using NCBI BLAST or the Rhabditina DB database (http://128.122.60.136/fmi/iwp/cgi?-db = RhabditinaDB&-loadframes) to identify the nematode strains. Primers used were: SSU18A (5′-AAAGATTAAGCCATGCATG) and SSU26R (5′-CATTCTTGGCAAATGCTTTCG) for 18S rDNA sequence amplification (Floyd *et al.,* 2002), and 5.8 S-1 (5′-CTGCGTTACTTACCACGAATTGCARAC) and KK28S-4 (5′-GCGGTATTTGCTACTACCAYYA MGATCTGC) for amplifying the ITS2 region (Kiontke *et al.,* 2011).

Collection and identification of wild LAB
To isolate wild LAB strains from the soil+fruit sample described above, a portion of each sample was mixed with water, vortexed, then centrifuged. The supernatant was serially diluted to 10^{-4} and then to 10^{-6}, and plated on MRS agar plates containing sodium azide and bromocresol purple (Yakuri, Japan) (0.05 g of each in 1 l media). Sodium azide inhibits growth of gram-negative bacteria, and bromocresol purple is a pH indicator that allows identification of LAB colonies by the color change of the surrounding agar from purple to yellow, due to lactic acid production. For molecular identification of LAB isolates, a fragment of 16S rDNA sequence was PCR amplified using the following primers: 27 F forward primer (5′-AGAGTTTGATCMTGGCTCAG) and 1492 reverse primer (5′-TACGGYTACCTTGTT ACGACTT).

Bacterial culture and media

Culture media was prepared similarly to a previous study (Jyoti *et al.,* 2004). The composition of 1 L of minimal media (M), which is a modified MRS, is as follows: 5 g yeast extract (BD, Cat#212750) 0.1 g $MgSO_4 \cdot 7H_2O$, 0.05 g $MnSO_4 \cdot H_2O$, 2 g NaH_2PO_4, 1 ml Tween80, 15 g agar. When supplementing glucose, 6.3 g of glucose was added to the minimal media, and for supplementing citrate, 2.2 g of sodium citrate was used according to previous protocols (Jyoti *et al.,* 2004). For fruit media, fresh fruit either directly from the above farms or store-bought was used for yuzu and apple media. For tomato media agar, organic tomato paste (Costco, Seattle, WA, USA, Item 633564) was used. For barley malt media agar, barley malt (BD, Cat#218630) was used. Whole yuzu fruits, apples or tomato paste were pureed with equal volumes of distilled water in a kitchen blender, and centrifuged at 8500 r.p.m. in a high speed centrifuge (Hanil Supra 30 K, Korea) in 500 ml bottles. The supernatant was buffered to pH 6.5 for optimal LAB growth and 2% agar was added and then autoclaved at 121 °C for 5 min. The agar media was then poured into plates. For barley malt media, 1.5% agar was added to barley malt and autoclaved.

Behavior assays

Odortaxis assays were performed on 9 cm petri dishes containing 1.6% Difco granulated agar (BD, Cat#214530) similar to the previously published methods (Bargmann *et al.,* 1993) except with some adjustments. A 7.5-mm agar plug from a media plate was made by using the back end of a 1000-µl disposable micropipette tip. An agar plug without bacteria was placed on one side of the lid of a 9-cm petri dish. An agar plug of the same type of media plate with bacteria was placed on the other side of the same lid. Two microliters of 1M sodium azide was placed directly below the agar plugs on the surface of the plate to paralyze the worms upon reaching the area of the plate directly below the agar plug. A population of adult worms were placed onto the origin of the assay plate and allowed to move freely on the plate for an hour. For chemical odor assays, 2 µl of diacetyl (Sigma, St Louis, MO, USA, 1:1000 dilution in ethanol) and 2 µl of benzalde-hyde (Sigma, 1:200 dilution in ethanol) was used. A 2.5-cm square box surrounding each spot of diacetyl was drawn, and attraction index was calculated as ((# of worms at the odor) − (# of worms at the control side))/(total # of worms).

For odor adaptation assays, methods similar to previous methods (Colbert and Bargmann, 1995) were used. Briefly, populations of adult worms were incubated in either a solution of 1:5000 dilution of diacetyl or 1:5000 dilution of benzaldehyde in S-basal buffer for 80 min. Worms were washed three times in S-basal, and subjected to the attraction assays described above.

Bacteria colony counts

An agar plug containing bacteria after 2 days of growth at 37°C from each of the media was placed in 0.85% NaCl, and then diluted to 10^{-5}, 10^{-6} and 10^{-7}. One-hundred microliters of these dilutions were plated on a 9-cm MRS agar plate overnight in duplicate, then counted. The trials were repeated on three separate days for Min and Min+Citr, four separate days for Min+Glu, five separate days for MRS and six separate days for Min+Glu+Citr.

Quantification of diacetyl

Concentration of diacetyl was quantified by modify-ing a spectrophotometric assay previously described (Mattessich and Cooper, 1989). Briefly, a solution containing 325 µl of solution A (saturated solution of creatine), 150 µl of solution B (3% NaOH, 3.5% α-naphthol) and 275 µl of distilled water was prepared, to which an agar plug with LAB was added and vortexed to release diacetyl that may have seeped into the agar. The mixture was spun down at 10,000 r.p.m. for 1 min and the supernatant was passed through a 0.25-µm membrane filter (Advantec, Toyo Roshi Kaisha, Japan) to eliminate any precipitates that may have formed. The reaction was left to develop for 1 h, and 100 µl of the solution was each added to three wells in a 96-well plate. Thus, each sample was performed in triplicate in each experiment. Absorbance at 525 nm was measured using a spectrophotometric plate reader (Infinite M200Pro, Tecan, Switzerland). Fresh solution B was prepared for each assay. Using commercially available diacetyl, we produced stan-dard curves for diacetyl concentration (Supplementary Figure S2), and then measured the concentration of diacetyl produced by LAB from the agar plug of various types of media. Each data point from each experiment is an average of the triplicate samples.

Results

L. paracasei produces diacetyl in the presence of citrate

It is well known in the food industry that LAB strains will produce the volatile chemical diacetyl when grown on media containing citrate (Michaelian *et al.*, 1933; Figure 1b). Citrate provides an extra source of pyruvate for lactic acid producing bacteria, and shifts sugar metabolism to allow the production of the buttery odor diacetyl as an oxidized by-product (Hugenholtz, 1993). Food producers use this ability of LAB to produce diacetyl to flavor fermented foods such as cheeses, yogurts and wine (Marshall, 1987; Bartowsky and Henschke, 2004; Smit *et al.,* 2005). As *C. elegans* is attracted to dozens of odors tested in the laboratory including diacetyl (Bargmann *et al.,* 1993), we asked whether diacetyl produced by LAB can be sensed and induce attraction behavior in *C. elegans*. We chose to use a strain of *Lactobacillus paracasei*, which can be found in plants, many dairy
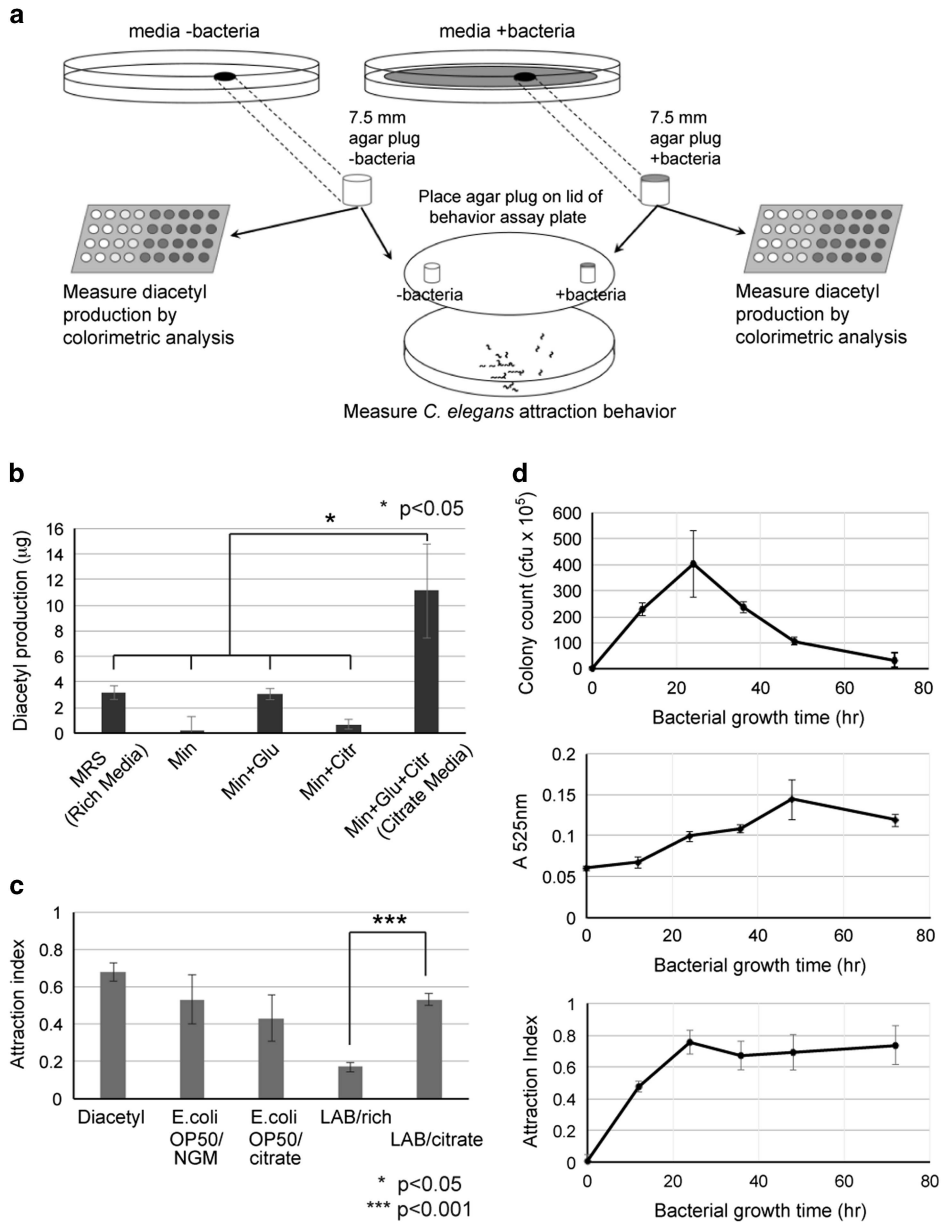
Figure 1 *C. elegans* is attracted to lactic acid bacteria grown on media containing citrate. (**a**) Strategy of study. *L. paracasei* (LAB) or *E. coli* strain OP50 is grown on several types of media (media +bacteria) including a rich MRS media (rich), a minimal media (min), minimal media containing glucose (min+glu), minimal media containing citrate (min+citr), minimal media containing both glucose and citrate (min+glu+citr) and nematode growth media (NGM). Media without bacteria (media – bacteria) is used as a control. A 7.5-mm agar plug is taken from each plate and used either for colorimeteric analysis to measure diacetyl production, or attraction behavior assay. In the attraction behavior assay, the +bacteria agar plug is placed on the lid of a 9-cm plate, and the –bacteria agar plug is placed on the opposite side, and *C. elegans* are allowed to move freely on an agar assay plate (see Materials and Methods). (**b**) Diacetyl production of *L. paracasei* grown for 2 days on different types of media. Diacetyl concentration is measured by colorimetric analysis in four separate experiments (Diacetyl standard curves are provided in Supplementary Figure S2). Error bars indicate s.e. (**c**) *C. elegans* attraction behavior to diacetyl (1:1000 dilution), *E. coli* strain OP50 or *L. paracasei* (LAB) grown for 2 days on rich or citrate media. Error bars indicate s.e. (**d**) Bacterial colony count (top), diacetyl production (middle) and *C. elegans* attraction behavior (bottom) to LAB grown on citrate media over 72 h. Colony count is measured as colony forming units (cfu). Diacetyl levels are measured as sample absorbance at 525 nm. Error bars indicate s.e. Significance was calculated by Student's *t*-test and *P*-values are indicated.

products and as a commensal in the human gut. It is also commonly used as a probiotic and is known to produce diacetyl (Aunsbjerg *et al.*, 2015). For this study we use *L. paracasei* and the term 'LAB' interchangeably.

First, we tested the ability of *L. paracasei* to produce diacetyl when grown in different nutrient conditions. We grew LAB for 48 h on several types of agar media: a rich MRS media, a minimal (M) media alone, or a minimal media added with either glucose,

citrate or glucose and citrate (Figure 1b). Then we took a 7.5-mm cylindrical agar plug from media plates with or without bacteria, and measured diacetyl levels by using a previously published spectrophotometric method for diacetyl concentration (Figure 1a) (Mattessich and Cooper, 1989). We found that LAB grown on rich media produced ~ 3 µg of diacetyl, whereas LAB grown on minimal media produced very little (Figure 1b). Supplementing the minimal media with citrate alone had little effect in diacetyl production, whereas supplementing with glucose increased production to about 3 µg. However, when the media was supplemented with both glucose and citrate, there was a 15-fold increase in diacetyl production compared with citrate alone, and a 3.5-fold increase compared with glucose alone or rich media. This was not due to differential growth and the number of bacteria in the different media, as there seemed to be very little correlation between diacetyl production and bacteria counts (Supplementary Figure S1). For example, although the average cell counts of LAB in the rich media and the minimal media supplemented with glucose and citrate were similar, diacetyl production was 3.5-fold higher in the latter (Supplementary Figure S1 and Figure 1b). Thus, LAB growth in the presence of both glucose and citrate (hereby referred to as 'citrate media') leads to a substantial production of diacetyl.

C. elegans is attracted to L. paracasei grown on citrate media

We next asked whether *C. elegans* is more attracted to LAB that produce diacetyl. We modulated previously published methods (Ward, 1973; Bargmann *et al.,* 1993) to assess *C. elegans* attraction to 7.5 mm agar plugs with or without bacteria for each type of media. As shown previously (Bargmann *et al.,* 1993; Sengupta *et al.,* 1996), worms showed strong attraction to commercially available chemical diacetyl and OP50 strain *E. coli,* the strain of bacteria that worms are normally cultured on in the laboratory (Figure 1c). When we tested odor attraction to bacteria, adult worms that were cultivated on NGM seeded with OP50 naturally were attracted to their familiar culture conditions, but also moderately attracted to OP50 when grown in citrate media. LAB, on the other hand, was minimally attractive when grown in rich media. Interestingly, however, when LAB was grown on citrate media, worms showed a strong attraction similar to OP50 strain *E. coli* grown on NGM. Thus, *C. elegans* can sense a difference in *L. paracasei* grown in the presence of citrate.

It is no surprise that *C. elegans* can smell bacteria (Harris *et al.,* 2014), and one possibility is that worms are simply sensing a large growth of LAB on citrate. Although we showed that LAB cell counts in the rich media and the citrate media did not correlate with their respective attraction index to worms (Supplementary Figure S1), we investigated this

possibility further. We tracked LAB growth on citrate media for 72 h, and measured bacterial growth, diacetyl production, and *C. elegans* attraction behavior (Figure 1d). LAB growth on citrate media peaked at 24 h, decreased considerably by 48 h and by 72 h most of the bacteria had died (Figure 1d, top). On the other hand, diacetyl production and accumulation continued for 48 h, and levels remained high even after 72 h (Figure 1d, middle). *C. elegans* attraction increased for 24 h and remained high even after 72 h, similar to the trend observed in diacetyl production (Figure 1d, bottom). Thus, *C. elegans* attraction to *L. paracasei* grown on citrate media correlates with diacetyl production, rather than bacterial growth patterns.

Adaptation to diacetyl inhibits attraction to L. paracasei grown on citrate

Is *C. elegans* actually able to distinguish between diacetyl-producing and non-producing *L. paracasei*? One way to test this is to take advantage of the ability of *C. elegans* to adapt to the odor diacetyl. Worms are strongly attracted to diacetyl, but after about 1 h pre-exposure to diacetyl, worms will adapt to the odor and ignore it (Colbert and Bargmann, 1995). We therefore pre-exposed worms to diacetyl for 80 min, and then assessed whether attraction to LAB grown on citrate media was attenuated. As expected, worms pre-exposed to diacetyl no longer showed attraction to diacetyl on an odortaxis assay. Interestingly, adaptation to diacetyl also resulted in the disappearance of attraction to LAB grown on citrate (Figure 2a).

Attractive odors are sensed by two pairs of sensory neurons at the anterior of *C. elegans*, called the AWA and AWC neuron pairs (Bargmann *et al.,* 1993). The AWA neurons are responsible for the attraction to diacetyl, whereas the AWC neurons sense other odors such as benzaldehyde, a chemical with an almond scent. Adaptation to benzaldehyde is also mediated by the AWC neurons. Thus, adaptation to benzaldehyde is completely independent of adaptation to diacetyl. To test whether the attenuation of attraction to LAB/citrate was specific for the diacetyl odor adaptation, we tested whether benzaldehyde pre-exposure could affect LAB/citrate attraction. As shown in Figure 2a, benzaldehyde pre-exposure resulted in adaptation behavior to benzaldehyde odortaxis. However, benzaldehyde pre-exposure had no effect on attraction to LAB grown on citrate. Thus, the adaptation response to *L. paracasei* was specific for the odor diacetyl. This strongly suggests that attraction to *L. paracasei* in *C. elegans* is mediated by diacetyl production in LAB.

The diacetyl receptor ODR-10 mediates attraction to L. paracasei

The adaptation experiments indicate a behavioral specificity for attraction to *Lactobacillus* that

produce diacetyl. We sought to provide molecular and genetic specificity for attraction to *Lactobacillus*. From mammals to nematodes, odors are sensed by membrane-bound G-protein coupled receptors (GPCRs). There are over a thousand GPCR-encoding genes in the *C. elegans* genome. However, in seminal work from the Bargmann lab, the odor receptor responsible for sensing diacetyl was identified as ODR-10 (Sengupta *et al.*, 1996). Mutants of *odr-10* are not attracted to diacetyl. We asked whether ODR-10 was also mediating the attraction to

Figure 2 *C. elegans* is attracted to diacetyl produced by LAB grown on citrate media. (**a**) Pre-exposure to the odor diacetyl results in adaptation behavior towards LAB grown on citrate media. Wild-type *C. elegans* was pre-exposed to 1:5000 dilution diacetyl, 1:5000 dilution benzaldehyde or a control buffer for 80 min, then performed an attraction assay either to diacetyl, LAB grown on citrate media or benzaldehyde. Error bars indicate s.e. Significance was calculated by Student's *t*-test and *P*-values are indicated. (**b**) Attraction was then tested to diacetyl, OP50 on citrate media, or LAB on citrate media with wild-type *C. elegans* and *odr-10(ky32)* mutants carrying a mutation in the ODR-10 diacetyl receptor. Error bars indicate s.e. Significance was calculated by Student's *t*-test and *P*-values are indicated.

L. paracasei grown on citrate media. In *odr-10(ky10)* mutants, attraction to diacetyl was significantly lower than wild-type N2 animals (Figure 2b). Interestingly, we saw a similar loss of attraction to *Lactobacillus* on the citrate media in *odr-10* mutants (Figure 2b). Thus, these experiments provide behavioral, genetic and molecular evidence that *C. elegans* is attracted to diacetyl produced by *L. paracasei*.

Both Caenorhabditis nematodes and LABs inhabit rotting fruit in nature
The most obvious source of citrate in nature is in fruits. Coincidently, one of the common places to find *C. elegans* and other nematodes is within rotting fruits (Felix and Braendle, 2010; Felix and Duveau, 2012). We wanted to confirm this previous finding locally in the Korean peninsula. We collected rotten fruit and adjacent soil from several fruit farms during each fruit harvest season. In the laboratory, we picked out nematodes from within these rotten fruit samples, and identified the nematodes by 18S rDNA sequencing. We successfully found *Caenorhabiditis* species such as *C. remanei* and *C. briggsae* in pear and yuzu fruit samples (Table 1). We also found other types of nematodes such as *Panagrellus* and *Pristionchus* that were also previously reported to be found in fruit (Felix and Duveau, 2012).

LABs are fermentative bacteria that are well known to ferment many types of foods including fruits and vegetables. We asked whether any LABs were among the species of bacteria residing in our rotten fruit samples. Thus, we isolated bacteria from our rotten fruit samples and identified LAB strains by 16S rDNA sequencing. From these samples we found members of the genera *Lactobacillus* and *Lactococcus* bacteria in rotten apple, pear, peach and yuzu fruit (Table 1). Taken together, we conclude that LABs and *Caenorhabditis* nematodes co-inhabit rotting fruit.

C. elegans is attracted to Lactobacillus fermenting citrus fruit media
It was already known that adult *C. elegans*, as well as other nematodes, could be found in relatively large populations in and near rotting fruits (Felix and Braendle, 2010; Kiontke *et al.*, 2011). However, it is
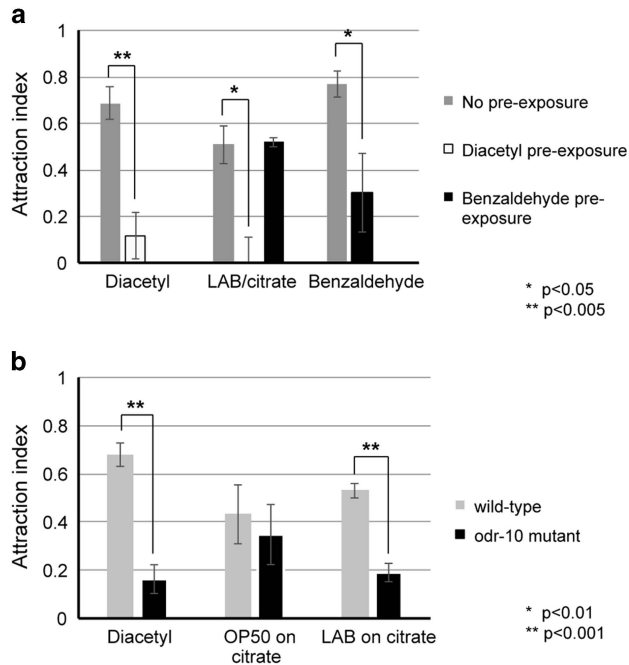
Table 1 Nematodes and LAB isolated and identified from rotten fruit

Fruit	Location	Date	LAB identified	Nematode identified
Apple	Chungju, S. Korea	Oct 2013	*Lactobacillus* sp. AS	Not sampled
Pear	Wonju, S. Korea	Oct 2013	*Lactobacillus* sp. PS03 *Lactobacillus* sp. PS04	Not sampled
Peach	Wonju, S. Korea	Aug 2014	*Lactobacillus* sp. D4 *Lactobacillus* sp. D5	Not sampled
Pear	Wonju, S. Korea	Oct 2014	*Lactobacillus* sp. PC04 *Lactococcus lactis* PB1 *Lactobacillus plantarum* PB2	*Pristionchus* sp. *Panagrellus* sp. *C. briggsae* strain PC01
Yuzu	Goheung, S. Korea	Nov 2014	*Lactobacillus* sp. Y091 *Lactobacillus* sp. Y092 *Lactococcus lactis* YS252	*C. remanei* strain Y01 *Pelodera* sp.

Rotten apple, peach, pear and yuzu were sampled from four locations. Pear was sampled once in 2013 and once in 2014. Genus or species identification is based on partial sequencing of 16S or 18S rDNA genomic sequencing.

not necessarily known why *C. elegans* prefers to reside in rotting fruit, or how they arrived there. *Caenorhabditis* and closely related nematodes are not likely to draw much nutritional benefit from non-microbial matter (Houthoofd *et al.*, 2002). One possibility is that *C. elegans* may be attracted to rotting fruit by the abundant and diverse populations of bacteria that ferment and use the sugars in fruit. *Caenorhabditis* and closely related nematodes are known as enrichment-type nematodes that grow and reproduce quickly to enormous numbers in the presence of quality bacterial food sources. We hypothesized, then, that the diacetyl attraction behavior we observed was actually a natural ecological behavior for *C. elegans* to find a large and growing source of bacterial food, sort of a 'bacterial bonanza', and that diacetyl acts as a cue for worms to track down the bacterial bonanza.

To investigate this hypothesis, we needed to find out whether LABs could produce diacetyl while fermenting citrate-containing fruits. To do this, we made several types of agar media using different fruits and a grain vegetable, a relatively common technique to culture LAB (Elliker and Hannesson, 1956). We used three different fruit-based media: yuzu, tomato and apple. Yuzu fruit (*Citrus medica L.*) is a citrus fruit native to East Asia that contains very high levels of citrate, and is grown agriculturally on the southern tip of South Korea. Tomato, although not a citrus fruit, contains some citrate. Apple is an acidic fruit that contains high levels of malic acid, but low levels of citrate. As a negative control, we used barley malt that contains little or no citrate. We grew *L. paracasei* on each of these fruit/vegetable media plates for 2 days, and then measured diacetyl production by spectrophotometric analysis. As seen in Figure 3a, LAB grown on the tomato, apple and barley media produce little diacetyl. In contrast, LAB grown on the yuzu media produces considerable levels of diacetyl albeit less than on citrate media.

We then assayed *C. elegans* attraction to LAB grown on each of the fruit/vegetable media. *L. paracasei* grown on barley malt media produces little diacetyl (Figure 3a). We found, though, that *C. elegans* is highly attracted to LAB on barley malt media (Figure 3b). However, this attraction is not dependent on the diacetyl receptor, as *odr-10* mutants showed significant attraction to barley-grown LAB (Figure 3b, black bars). Thus, other odors may be mediating this attraction. Yuzu fruit, on the other hand, contains high levels of citrate, and *L. paracasei* grown on yuzu media produces high levels of diacetyl (Figure 3a). When we tested attraction of wild-type worms to LAB grown on yuzu, we found that it was highly attractive, but strikingly, very little attraction was seen in the *odr-10* mutants, indicating that this attraction was mediated by the diacetyl receptor. Growth on apple and tomato media results in low or moderate levels of diacetyl. Consistent with this,
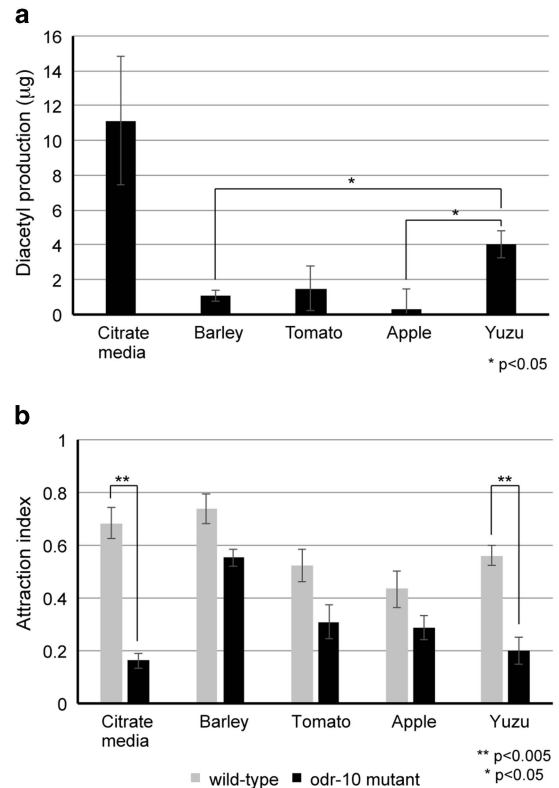
Figure 3 *C. elegans* is attracted to diacetyl produced by LAB grown on media made from the citrus fruit yuzu. LAB was grown for 2 days on media made from apple, tomato, barley malt, or yuzu, and diacetyl production (**a**) or attraction behavior of wild-type and *odr-10* mutants (**b**) of an agar plug from the plates was assessed. Diacetyl standard curves (Supplementary Figure S2) were used to calculate diacetyl levels. Error bars indicate s.e. Significance was calculated by Student's *t*-test and *P*-values are indicated.

both cause a moderate attraction to worms, and this is partially but not significantly mediated by ODR-10. Thus, diacetyl can mediate attraction to *Lactobacillus* that are fermenting citrus fruit media.

Wild Caenorhabditis is attracted to wild LAB strains isolated from yuzu

We have demonstrated that both Rhabditid nematodes and LAB can be found in fruits, and diacetyl produced by an LAB grown on citrate or citrus fruit media is attractive to *C. elegans*. However, whether this phenomenon of odor attraction between nematodes and LABs occurs in nature is not known. We wanted to test whether an LAB isolated from rotting fruits in nature produced diacetyl, and whether wild nematodes isolated from the same rotting fruit are attracted to the LAB. We first tested whether *C. elegans* was attracted to a wild LAB strain. We used the *Lactobacillus* strain Y091 that we isolated from yuzu fruit (Table 1), grew it for 2 days on citrate and yuzu media, and tested whether *C. elegans* was attracted to it. As shown in Figure 4a, wild-type *C. elegans* was attracted to the wild LAB grown on
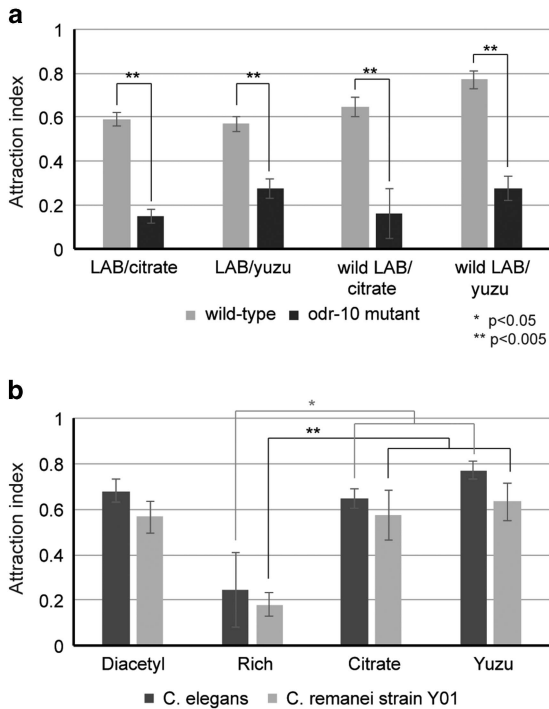
Figure 4 Wild *Caenorhabditis* strain is attracted to wild LAB strains isolated from yuzu. (**a**) Attraction behavior of wild-type and *odr-10* mutant *C. elegans* to wild *Lactobacillus* strain Y091 isolated from rotten yuzu was assessed, when grown on either citrate media or yuzu media. (**b**) Attraction behavior of wild-type *C. elegans* and wild *C. remanei* strain Y01 isolated from rotten yuzu to wild *Lactobacillus* strain Y091 was assessed. Wild LAB was grown on either citrate or yuzu media. Error bars indicate s.e. Significance was calculated by Student's *t*-test and *P*-values are indicated.

either media. To test whether this attraction was mediated by diacetyl sensation, we tested *odr-10* mutants for attraction behavior. Worms that lack the diacetyl receptor were no longer attracted to the wild LAB grown on either citrate or yuzu media. Thus, *C. elegans* attraction to a wild LAB strain isolated from yuzu is mediated by diacetyl sensation.

All nematodes including *C. elegans* use their sense of smell to track cues in the environment. In fact, many other nematodes including the closely related Rhabditid nematode *Caenorhabditis remanei* is attracted to the odor diacetyl (Hong and Sommer, 2006). We isolated a wild strain of *C. remanei* from rotten yuzu (Table 1), and tested its attraction to diacetyl. As shown in Figure 4b, this wild strain was attracted to diacetyl, similarly to the laboratory strain of *C. remanei* (Hong and Sommer, 2006). Next, we asked whether this wild *C. remanei* was attracted to the wild LAB strain Y091 that was also isolated from yuzu fruit. Y091 wild LAB grown on either citrate or yuzu was highly attractive to wild *C. remanei* isolate from yuzu, but not attractive when grown on rich media (Figure 4b). Taken together, these data support the hypothesis that nematodes sense diacetyl produced by LAB growing on citrus fruit media.

Discussion

LAB have been well studied due to their importance in the food industry and the probiotic benefits they confer towards human health. However, little is known about the ecology of LABs in nature outside of the human gut. Likewise, *C. elegans* has been one of the most useful laboratory genetic model organisms over the last 40 years, but little is known about natural interactions with *C. elegans* in nature. Here, we employed diverse biological methods and attempted to quantify a natural ecological phenomenon in the laboratory using *L. paracasei* and *C. elegans* genetics. We showed that *C. elegans* is attracted to *L. paracasei* grown on citrate or yuzu media. We demonstrated that the attraction to the LAB arises specifically from diacetyl odor signaling. This phenomenon was not limited to *C. elegans* and *L. paracasei*, but wild *Caenorhabditis* isolated from rotten yuzu fruit also shows a robust attraction to *Lactobacillus* isolated from rotten yuzu fruit grown on citrate media or yuzu media. We have described a genetic and molecular basis for an ecological interaction between bacteriovorous nematodes and bacteria in rotten fruit. These experiments also establish a natural role for odor behaviors and sensory circuitry in an animal.

Our hypotheses are that (1) first, LAB grows on fresh citrus fruit, (2) LAB metabolize sugar utilizing citrate in the fruit, (3) diacetyl is produced as a by-product, (4) nematodes sense diacetyl, activating diacetyl odor receptors, (5) nematodes are attracted to diacetyl and navigate to the source of LAB, (6) nematodes find a diverse bacterial bonanza, lay eggs and quickly reproduce.

The study of olfactory circuitry and odor behaviors in an animal with a simple and intact nervous system like *C. elegans* has been invaluable in understanding odor discrimination, odor memory and adaptation, and integration with other sensory systems. However, this study is the first demonstration to our knowledge of a natural odor attraction between *C. elegans* and a specific odor released by bacteria found in the nematode's environment. In addition, we show that after pre-exposing the worms to chemical diacetyl without bacteria, *C. elegans* adapts to the odor and ignores the diacetyl emanating from the LAB during the attraction assay. It is thought that in nature odor adaptation is a behavioral mechanism for worms to ignore profitless odors and focus on odors that lead the worm to food. Diacetyl production is long-range volatile cue that may lead the worms to the area of LAB growth, but LAB itself may not be a preferable source of nutrition for *C. elegans*. This could lead to adaptation to diacetyl. One speculation is that adaptation to diacetyl may help focus the worm on searching for nearby sources of preferred bacteria. Overall, these experiments attribute ecological significance for odor attraction behavior and memory in nematodes.

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We speculate that many other odors emanate from countless other microorganisms that they interact with and may account for the large number of chemosensory receptors that have evolved within the *Caenorhabditis* genomes (Robertson, 1998; Thomas and Robertson, 2008). In fact, we observe diacetyl receptor-independent attraction to LAB grown on barley, tomato and apple, indicating odors other than diacetyl are mediating attraction in these assays. Furthermore, a recent investigation has suggested the importance of the diacetyl receptor for *C. elegans* to sense *E. coli* bacteria, allowing males to make correct behavioral choices between eating and mating (Ryan *et al.,* 2014). Attraction to diacetyl odor in *C. remanei* and the closely related nematode *Caenorhabditis briggsae* has been established (Hong and Sommer, 2006). In this study, we demonstrate that wild *C. remanei* isolated from rotten yuzu is attracted to LAB grown on citrate or yuzu media. Whether a homolog of ODR-10 in *C. remanei* mediates this attraction requires further study.

Although we demonstrated an attraction to diacetyl emanating from a LAB in rotten fruit by nematodes in laboratory conditions, whether diacetyl sensation allows nematodes to increase nutrition and acquire any reproductive fitness advantages requires a broader study. In fact, our observations confirm other studies that show *C. elegans* grown solely on *Lactobacillus* develop slowly and become calorically restricted (Kim and Mylonakis, 2012; Zhao *et al.,* 2013). However, LABs are only one type of bacteria out of many that reside in rotten fruit. In further experiments, we identified two soil bacteria, *Enterobacter sp.* and *Arthrobacter sp.*, in the intestines of wild *C. remanei* strain Y01 that we recovered from rotten yuzu fruit (data not shown). In addition, we found that wild-type *C. elegans* produces moderate brood sizes when grown on *Arthrobacter sp.* and large brood sizes when grown on *Enterobacter sp.* comparable to the large brood sizes observed when grown on laboratory strain OP50 *E. coli* (data not shown). This shows that nematodes can find and consume various bacteria in fruit. Previous studies report that increased numbers of nematodes are found in areas of the soil that are populated with increased diversity of bacteria (Bongers, 1990; Darby *et al.,* 2007). Our hypothesis is that diacetyl acts as a cue to allow nematodes to locate large and diverse bacterial bonanzas growing in an area where nutrients for microbial growth are abundant.

The molecular and genetic studies of *C. elegans* and the microbiological and biochemical studies of LAB have contributed greatly to our understanding of basic biology and human health. Our work takes advantage of the breadth of knowledge accumulated in *C. elegans* and LAB research to identify how animals and microbes may interact in nature at the molecular level. Further study should elucidate other molecules that mediate ecological interactions between *C. elegans* and LAB and other microbial communities.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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