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Original Article

Identification of Odor Blend Used by *Caenorhabditis elegans* for Pathogen Recognition

Soleil E. Worthy[1], German L. Rojas[2], Charles J. Taylor[1] and Elizabeth E. Glater[2]

[1]Department of Chemistry and [2]Department of Neuroscience, Pomona College, Claremont, CA 91711, USA.

Correspondence to be sent to: Elizabeth Glater, Department of Neuroscience, Pomona College, Claremont, CA 91711, USA. e-mail: elizabeth.glater@pomona.edu

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Abstract

Animals have evolved specialized pathways to detect appropriate food sources and avoid harmful ones. *Caenorhabditis elegans* can distinguish among the odors of various species of bacteria, its major food source, but little is known about what specific chemical cue or combination of chemical cues *C. elegans* uses to detect and recognize different microbes. Here, we examine the strong innate attraction of *C. elegans* for the odor of the pathogenic bacterium, *Serratia marcescens*. This initial attraction likely facilitates ingestion and infection of the *C. elegans* host. Using solid-phase microextraction and gas chromatography coupled with mass spectrometry, we identify 5 volatile odors released by *S. marcescens* and identify those that are attractive to *C. elegans*. We use genetic methods to show that the amphid chemosensory neuron, AWC^{ON}, senses both *S. marcescens*-released 2-butanone and acetone and drives attraction to *S. marcescens*. In *C. elegans*, pairing a single odorant with food deprivation results in a reduced attractive response for that specific odor. We find that pairing the natural odor of *S. marcescens* with food deprivation results in a reduced attraction for the natural odor of *S. marcescens* and a similar reduced attraction for the synthetic blend of acetone and 2-butanone. This result indicates that only 2 odorants represent the more complex odor bouquet of *S. marcescens*. Although bacterial-released volatiles have long been known to be attractive to *C. elegans*, this study defines for the first time specific volatile cues that represent a particular bacterium to *C. elegans*.

Key words: food, odor mixture, olfaction, *Serratia marcescens*, solid-phase microextraction

Introduction

The nematode *Caenorhabditis elegans* lives in rotting fruit and plant matter, where it must navigate a complex environment containing some microbes which are nutritious food and some which are pathogenic (Félix and Duveau 2012). *Caenorhabditis elegans* can distinguish among the odors of various species of bacteria and shows strong innate preferences for particular bacterial species (Shtonda and Avery 2006; Ha et al. 2010; Harris et al. 2014). Although a few attractants released by specific microbes have been identified, it is unknown what specific chemical cue or combination of chemical cues *C. elegans* uses to recognize and detect different microbes (Beale et al. 2006; Niu et al. 2010; Werner et al. 2014; Choi et al. 2016; Hsueh et al. 2017). Is recognition of a natural bacterial odor blend based on several components, a few key components or a single unique component? Identifying these ethologically relevant chemical cues is essential to understanding the molecular basis of interactions between *C. elegans* and microbes.

Here, we examine how *C. elegans* detects the pathogenic bacterium, *Serratia marcescens*, because *S. marcescens* has been shown to influence the behavior of *C. elegans* in several ways. First, the odor of *S. marcescens* is highly attractive to *C. elegans* and this attraction likely facilitates consumption and subsequent intestinal infection

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that kills the worm after 2–3 days (Pujol et al. 2001; Mallo et al. 2002; Kurz et al. 2003; Zhang et al. 2005; Glater et al. 2014). Thus, *S. marcescens* likely releases volatiles that are attractive to *C. elegans*. Second, although *C. elegans* is initially attracted to *S. marcescens*, after several hours the worms leave the lawn of pathogenic bacteria. A secreted, non-volatile, natural bacterial product mediating this repulsion from specific strains of *S. marcescens* has been identified and is serrawettin W2, a cyclic lipodepsipentapeptide (Pradel et al. 2007). Third, the behavioral response of *C. elegans* to the odor of *S. marcescens* exhibits plasticity and is modified after the worm has encountered *S. marcescens*. After *C. elegans* is infected by *S. marcescens*, *C. elegans* avoids the odor of this specific pathogenic bacterium (Zhang et al. 2005). Fourth, attraction to *S. marcescens* is also modified on an evolutionary timescale. It is likely that attraction for *S. marcescens* is a fast evolving trait because different strains of *C. elegans* isolated from different regions around the world show natural variation in preference for the odor of *S. marcescens* (Glater et al. 2014). In addition, in an experimental co-evolution study, *C. elegans* exhibited avoidance of *S. marcescens* after being passaged for 30 generations with the pathogen (Penley and Morran 2017). Therefore, we propose to examine 2 questions. First, what volatiles initially attract *C. elegans* to *S. marcescens*? Second, after the odor of *S. marcescens* is paired with an adverse experience, what volatiles are later used to recognize and avoid *S. marcescens*?

In this study, we define the attractive volatiles necessary for recognition of *S. marcescens* by *C. elegans*. We first identify the major volatiles released by *S. marcescens* using solid-phase microextraction and gas chromatography coupled with mass spectrometry and then determine which of these volatiles are attractive. We use genetic methods to determine the sensory neurons necessary for detection of *S. marcescens* and *S. marcescens*-released volatiles. Last, we use aversive conditioning assays in which the odor of *S. marcescens* is paired with food deprivation and then animals are tested for chemotaxis to individual odorants released by *S. marcescens*, synthetic mixtures of these odorants, or the natural odor of *S. marcescens*, to determine what component or components best represent *S. marcescens*. Taken together, we determine that 2 odorants, acetone and 2-butanone, are sufficient to represent *S. marcescens* to *C. elegans* and are detected by the AWC^{ON} sensory neuron.

Materials and methods

Nematode growth and strains

Strains were grown and maintained under standard conditions at 20°C on nematode growth media (NGM) (Brenner 1974). Animals were grown on plates seeded with *Escherichia coli* HB101 ATCC 33694. Some strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440), Dr. Cornelia Bargmann, Dr. Sreekanth Chalasani, Dr. Elissa Hallem and Dr. Paul Sternberg. CX4 *odr-7(ky4) X* (Sengupta et al. 1994), CX3938 *lim-4*(*ky403*) *X* (Sagasti et al. 1999), PR680 *che-1(p680) I* (Dusenbery et al. 1975), CX4651, *osm-9(ky10) ocr-2(ak47) V* (Tobin et al. 2002), CX6339 *ceh-36(ky640) X* (Lanjuin et al. 2003), CX6161 *nsy-5(ky634) I* (Chuang et al. 2007), CX10232 *nsy-7(tm3080) IV* (Lesch et al. 2009), CX9190 *nsy-1(ky542) II* (Wes and Bargmann 2001), EAH2 *gcy-9 (tm2816) X* (Carrillo et al. 2013), CX13078 *tax-4(p678) III* (Dusenbery et al. 1975), CX13790 *tax-4(p678); kyEx4233* (*ceh-36*::tax-4 sl2::GFP* and *myo3::mCherry*), CX13359 *tax-4(p678); kyEx3945* (*sra-9::tax-4 sl2::GFP* and *elt-2::mCherry*) (Macosko et al. 2009), CX11576 *kyEx3097* (*sra-9::TeTx::mcherry* and *elt-2::GFP*) (Macosko et al. 2009), CX5868 *kyIs140 I* (*str-2::GFP*); *kyEx613* (*odr-3::TeTx*; *odr-1::RFP* and *elt-2::GFP*) generated by Alvaro Sagasti, CX13458 *nsy-7(tm3080); kyEx2125 (odr-3p::nsy-7* and *ofm-1::dsRed*), PS5716 *tax-4(p678); syEx1045 (gcy-33::tax-4 and myo-2::gfp)* (Hallem and Sternberg 2008).

Transgenes

Extra chromosomal transgenes were made by injection of DNA plasmids and a fluorescent coinjection marker into the gonads of young adult hermaphrodites. Two to 3 independent lines from each injection were assayed. DNA was cloned into *psm* vector. AWC *ceh-36p** ends: ctcacatccatctttctggcgactgtttcatt…gcctgcccccgcatgcacaa; ASK *sra-9p* ends: gcatgctatattccaccaaa....tgtgcatcaatcatagaaca; *tax-4* cDNA ends: atgtcaacggcggaacctgc.…ctgaatccttgctcaaatag; *odr-3p::nsy-7* cDNA as described (Lesch and Bargmann 2010); *sra-9p::TeTx::mCherry* as described (Macosko et al. 2009); and *odr-3p::TeTx* generated by Alvaro Sagasti.

Bacterial strains and reagents

Bacterial strains *S. marcescens* ATCC 274 and *E. coli* HB101 ATCC 33694 were obtained from the American Type Culture Collection.

Bacterial choice assay

The 2-choice bacterial choice assay was modified from Zhang et al. (2005). Briefly, bacteria were grown overnight in Luria Broth (LB) at 26°C, centrifuged, and then resuspended in LB at an OD600 of 1.0 for *S. marcescens* or an OD600 of 10 for *E. coli* HB101, and 25 µL of each bacterial suspension was spotted onto an NGM plate and incubated for 5 h at 20°C. At these OD600 values, both bacteria had approximately the same cellular density: at OD600 of 1.0, *S. marcescens* yields $2.1 \times 10^9 \pm 1.5 \times 10^9$ colony-forming units (cfu) per ml; at OD600 of 10, *E. coli* HB101 yields $3.2 \times 10^9 \pm 1.7 \times 10^9$ cfu per mL. Adult animals were washed 3 times in S-basal buffer and 50–200 animals were placed near the center of an NGM plate, equidistant from the 2 bacterial patches. Animals were allowed to move freely for 1 h, then 5 µL of 1 M sodium azide was added to each bacterial patch to immobilize worms on bacterial patches, and then worms were counted. The bacterial choice index is the number of worms on *S. marcescens* minus the number of worms on *E. coli* divided by the number of worms on both bacterial patches. For *E. coli* with acetone and 2-butanone assays, *E. coli* was prepared as above at OD600 of 10. Immediately before worms were placed on the assay plates, 2 µL of 1 mM 2-butanone diluted in ethanol and 2 µL of 10 mM acetone diluted in ethanol was pipetted on one *E. coli* patch and 4 µL of ethanol was pipetted on the other *E. coli* patch.

Chemotaxis assays

Chemotaxis assays were performed using 10 cm square chemotaxis plates as described (Tsunozaki et al. 2008). In brief, assay agar was 2% agar, 1 mM $MgSO_4$, 1 mM $CaCl_2$ and 5 mM phosphate buffer (pH 6.0). Chemical dilutions were in ethanol at the concentrations indicated in figure legends. 2 µL of diluted chemical were pipetted on one side of the plate, 2 µL of ethanol on the other side, and 2 µL of 1 M sodium azide on both sides to anaesthetize animals that reached odor or ethanol sources. For the chemical bouquet, diluted chemicals were combined and then 2 µL were pipetted on one side of the plate. Animals were washed 2 times in S-basal, once in water and then 50–200 placed in the center of the plate, and the distribution of animals counted after 1 h. The chemotaxis index is the number of worms on odor side minus the number of worms on diluent side divided by the total number of worms that have left the origin.

Aversive olfactory conditioning assays

The odor conditioning assays were modified from Bargmann and Colbert (1995). Adult worms were washed 3 times in S-basal and then transferred to an NGM plate without bacterial food (approximately 500 worms were transferred to each plate). For naïve animals, another NGM plate was used for the lid and inverted above the plate of worms. For the odor-conditioned animals, 4 spots of 3 μL neat chemical was pipetted on an NGM plate, the plate was used for a lid and inverted above the plate with worms. The 2 plates were sealed with parafilm and incubated for 90 min. Then the worms were assayed in chemotaxis assays described above to pure odorants or mixtures of odorants at concentrations indicated in figure legends. For bacterial-conditioned assays, naïve animals were prepared in the same way as for odor conditioned assays. For bacterially conditioned animals, *S. marcescens* bacteria was grown overnight in LB at 26°C and then resuspended (OD600 = 10). Five spots of 100 μL of the bacterial suspension was spotted onto an NGM plate and incubated for 5 h at 20°C. Then the NGM plate with *S. marcescens* bacteria was used for a lid and inverted above plate of worms on NGM plate without food, sealed with parafilm and incubated for 90 min. Then the worms were assayed in chemotaxis assays described above to pure odorants or mixtures of odorants at concentrations indicated in figure legends. For the odor of *S. marcescens*, 5 μL of *S. marcescens* (OD600 = 1.0) or 5 μL of LB media was pipetted on NGM agar plate and incubated at 20°C for 6.5 h. Then the agar plug with bacteria or media was cut out with a sterile blade and placed on the lid of a chemotaxis plate directly above 2 μL of 1 M sodium azide on the chemotaxis plate.

For all behavioral assays, data points are means of $n \geq 5$ assays with between 50–200 animals each, repeated on at least 2 different days.

Solid-phase microextraction gas chromatography mass spectrometry

Bacteria were prepared as for bacterial choice assay. Bacteria were grown overnight in LB at 26°C, centrifuged, and then resuspended at an OD600 = 10. Two NGM plates were prepared, each with 9 spots of 25 μL of bacterial suspension. For the controls, 25 μL of LB media without bacteria was spotted on NGM plate. Plates were incubated for 5 h at 20°C. Then 18 squares (each approximately 8 × 8 mm) of the NGM agar with 25 μL bacteria suspension or LB media were placed in a GC-MS glass vial at 20°C for 1 h (Supplementary Figure 1). Each agar square was extracted using a metal spatula that was sterilized by placing in ethanol, flaming, and allowing to cool at room temperature briefly before use. Samples were analyzed by SPME-GC-MS using the Agilent 6890 GC System equipped with a Restek, Rtx-5 column, and Agilent 5973 Mass Selective Detector. The temperature program was: hold 2 min at 35°C, increased to 140°C at a rate of 10°C/min, and then increased to 250°C at a rate of 100°C/min and hold at 250°C for 3 min. MS ranged from 30 to 550 in full scan mode. VOCs were identified with the NIST 11 (National Institute of Standards and Technology) mass spectral library and pure chemical standards run following the same parameters as for bacterial samples. Bacterial samples of *S. marcescens* were prepared 3 times on different days and then analyzed, bacterial samples of *E. coli* of were prepared twice on different days and then analyzed, and LB control samples were run immediately before or after each bacterial sample. To determine approximate absolute quantities of volatiles, pure chemicals of known quantity were run and the quantities of compounds in *S. marcescens* headspace were calculated relative to the total peak areas of the standards. All standard chemicals were at least 98% purity and were purchased from Sigma-Aldrich (USA).

Results

Identification of *S. marcescens* attractive volatiles

Although *S. marcescens* is a bacterial pathogen that can kill *C. elegans*, the odor of *S. marcescens* is more attractive to the wild-type *C. elegans* strain N2 than its standard laboratory food source, *E. coli* (Zhang et al. 2005; Glater et al. 2014). We evaluated olfactory preferences of *C. elegans* using a bacterial choice assay in which worms migrate to one of 2 patches of bacteria on opposite sides of an agar plate (Figure 1A) (Zhang et al. 2005). In this assay, the animals' first approach over 1–2 h is dominated by their olfactory preferences for volatile odors released by the bacteria and *C. elegans* shows a strong preference for *S. marcescens* over *E. coli* (Figure 1B).

We used solid-phase microextraction (SPME) gas chromatography-mass spectrometry (GC-MS) to identify the volatiles present in the headspace of *S. marcescens*. The headspace refers to the volume of air above the bacterial sample within the GC-MS glass vial. The bacteria were grown on agar plates in the same manner as grown

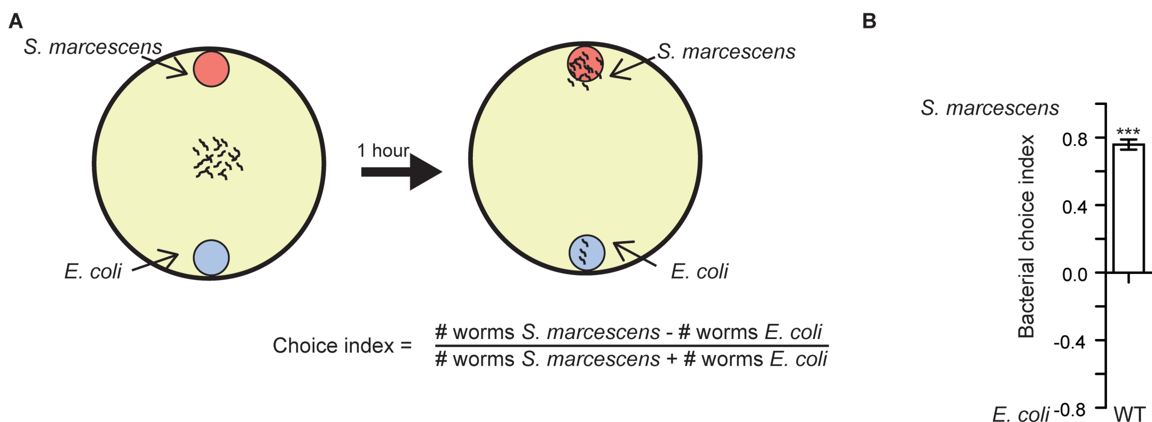

Figure 1. *Caenorhabditis elegans* prefers *Serratia marcescens* over *E. coli.* (**A**) Cartoon of the bacterial choice assay. Approximately 50–200 worms are placed on an agar plate between 2 patches of bacteria, which they can approach by olfactory chemotaxis. Animals were allowed to move freely for 1 h before plates were scored. Cartoon adapted from Glater et al. 2014. (**B**) Bacterial choice index of wildtype animals. WT show a significant preference for *S. marcescens* (OD600 = 1.0) over *E. coli* (OD600 = 10), *** indicates $P < 0.001$, one sample Student's *t*-test, compared to 0, $n = 25$ assays. Error bars represent SEM.

for bacterial choice assays (Supplementary Figure 1). We found consistently in 3 replicate samples made on different days that *S. marcescens* headspace contained 5 major volatile organic compounds (VOC) that were not present in the headspace of *E. coli* or the control sample, LB media spotted on NGM agar. We used the NIST 11 (National Institute of Standards and Technology) mass spectral library to identify tentatively the VOCs and confirmed the identity of the VOCs using standards made from pure chemicals. The unique VOCs identified in *S. marcescens* cultures were: acetone, dimethyl sulfide, 2-butanone, ethyl acetate, and dimethyl disulfide (Figure 2). Retention times of identified VOCs are in Supplementary Table 1.

We tested each of the candidate odorants in *S. marcescens* headspace at a range of concentrations in chemotaxis assays. Of the 5 VOCs, *C. elegans* showed a strong preference for 2-butanone and acetone at a range of concentrations, but weak or no preference for the other 3 odorants at most concentrations (Figure 3A and C). Ethyl acetate was weakly attractive at high concentrations of 1 M and undiluted stock (Figure 3D). Dimethyl sulfide and dimethyl disulfide were not significantly attractive at all concentrations tested (Figure 3B and E). These results are generally consistent with previous studies that have shown that 2-butanone is attractive at a range of concentrations, and that acetone is attractive when tested at 1:100 dilution (approximately 100 mM) (Bargmann et al. 1993). However, previous work has shown that 1:100 dilution of ethyl acetate (approximately 100 mM) and 1:100 dilution of dimethyl disulfide (approximately 100 mM) to be weakly attractive, but we see no attraction in our chemotaxis assays at these concentrations (Bargmann et al. 1993; Hsueh et al. 2017). This difference in chemotaxis indexes may be because we used square chemotaxis plates while these previous studies used round plates and the concentration gradient encountered by the worms may differ.

Next, we tested an odorant mixture of all 5 volatiles found in *S. marcescens* headspace at the approximate concentrations detected in the headspace (5 mM acetone, 0.5 mM dimethyl sulfide, 0.5 mM 2-butanone, 0.1 mM ethyl acetate, and 1 mM dimethyl disulfide, Figure 3F). As expected from the results of chemotaxis with single odorants, the bouquet of all 5 odorants was very attractive. Next, we tested different subsets of the odorants (Figure 3F). Acetone is the most attractive compound because the bouquet lacking acetone is significantly less attractive than the bouquet of all 5 volatiles, although this bouquet is still somewhat attractive. This remaining attraction is likely due to 2-butanone, the second most attractive compound in the mixture. The 3 remaining odorants do not contribute to the attractiveness of the bouquet because the bouquet of all 5 odorants is as attractive as the blend of only acetone and 2-butanone. Taken together, this indicates that 2-butanone and acetone are likely the major attractive volatiles in *S. marcescens* headspace.

Identification of sensory neurons that detect *S. marcescens* volatiles

In order to evaluate whether the attractive volatiles we identified are what *C. elegans* uses to recognize *S. marcescens*, we next determined which sensory neuron(s) detect *S. marcescens* bacterial odor and then whether these sensory neurons also detect the identified attractive volatiles, acetone, and 2-butanone. *Caenorhabditis elegans* has 2 pairs of olfactory neurons that sense attractive volatile molecules, AWC and AWA, and 3 pairs of chemosensory neurons that sense repulsive odors, AWB, ASH, and ADL. To determine which neurons are important in preference for *S. marcescens*, we tested well-characterized *C. elegans* mutants with defects in the cell fates of specific chemosensory neurons in bacterial choice assays (Figure 4A). Mutations that affected the AWC neuron exhibited defective choice behavior while other mutants exhibited wild-type choice behavior. The *odr-7* mutants and *lim-4* mutants, which have defects in AWA or AWB cell fates, respectively (Sengupta et al. 1994; Sagasti et al.

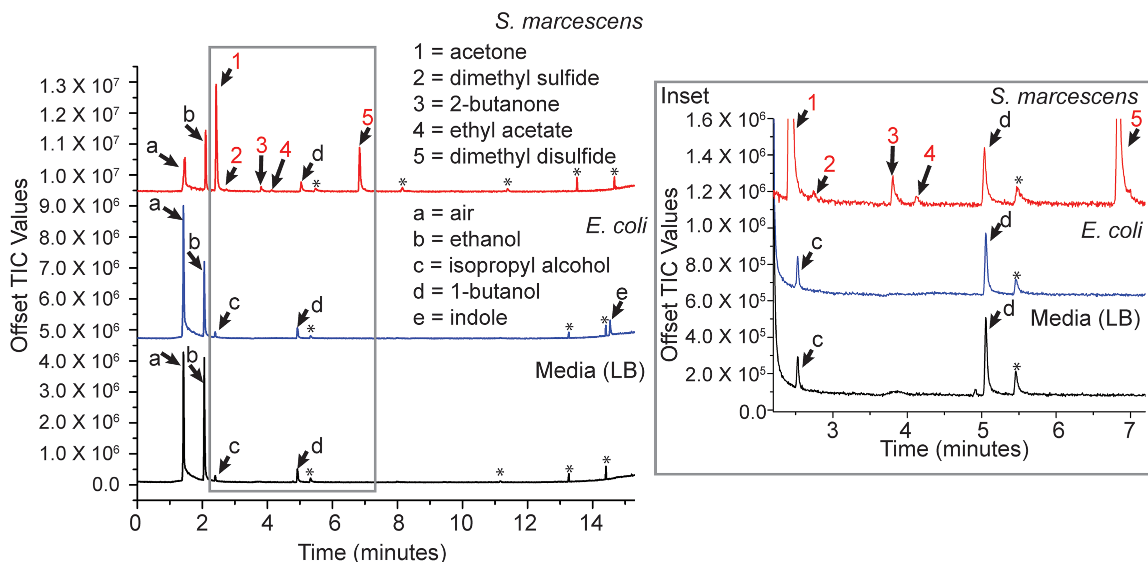

Figure 2. Gas chromatography-mass spectrometry of headspace of *Serratia marcescens*, *E. coli* and LB media control. (**A**) Overnight liquid cultures of bacteria were spotted on NGM agar plates and incubated for 5 h, NGM agar squares were placed inside a GC-MS glass vial for 1 h. Then a SPME fiber was inserted into the sample vial containing the bacteria to sample the volatiles in the headspace. Representative total ion chromatograms for *S. marcescens* (OD600 = 10) (top), *E. coli* (OD600=10) (middle), and LB media control (bottom). Peaks unique to *S. marcescens* are numbered and labeled: 1 = acetone, 2 = dimethyl sulfide, 3 = 2-butanone, 4 = ethyl acetate, 5 = dimethyl disulfide. Prominent other peaks not unique to *S. marcescens:* a = air, b = ethanol, c = isopropyl alcohol, d = 1-butanol, e = indole. Asterisks indicate "fiber peaks," volatile organic compounds released by the SPME fiber. Peaks were identified with NIST 11 (National Institute of Standards and Technology) mass spectral library and confirmed with known standards. See sample retention times in Supplementary Table 1. (**B**) Inset shows magnified chromatogram for approximate retention times 2–7 min. Peaks are labeled in the same manner as in (A).

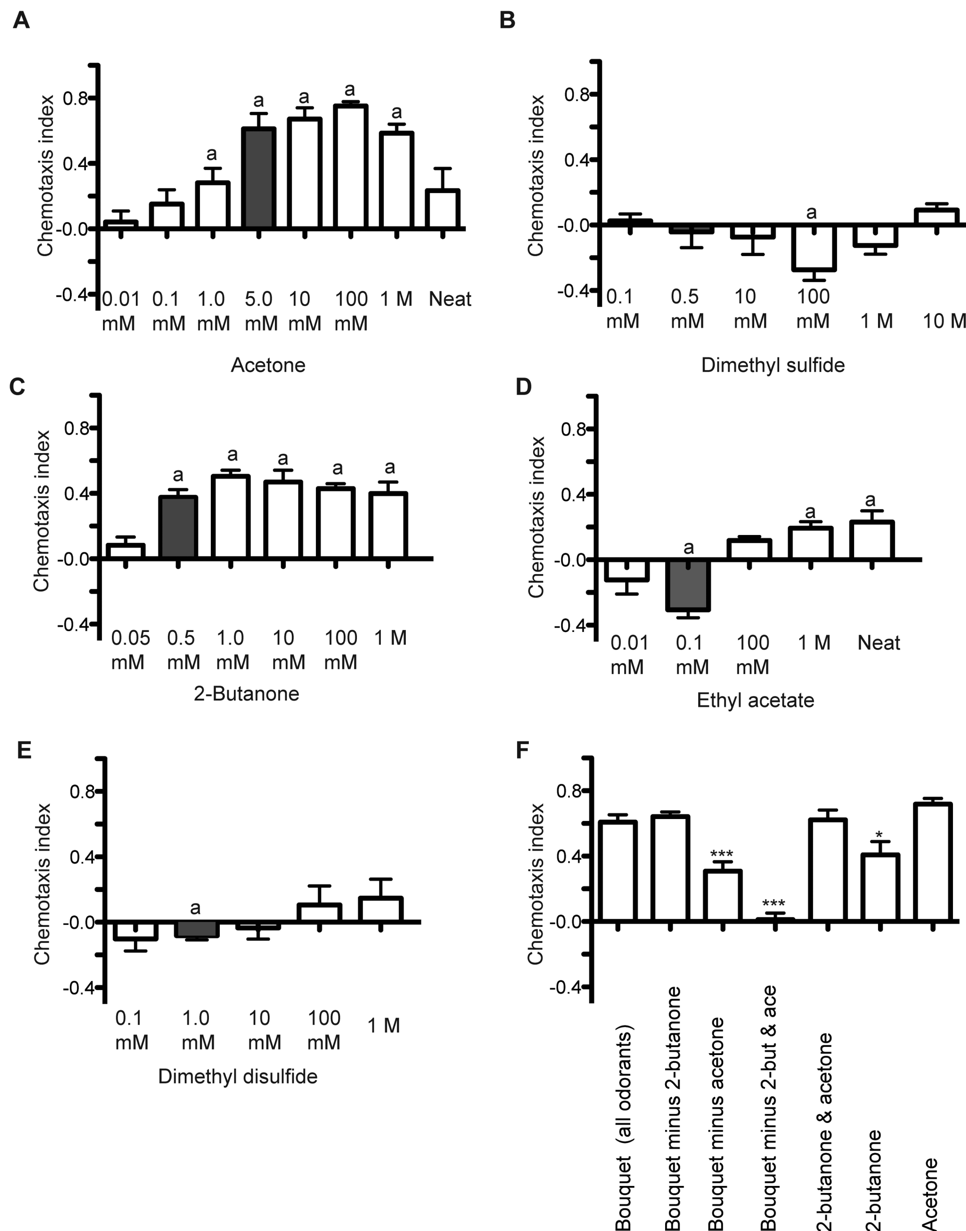

Figure 3. Major attractive volatiles released by *Serratia marcescens* are acetone and 2-butanone. Chemotaxis to different concentrations of identified volatiles in *S. marcescens* headspace. All volatile organic compounds (VOCs) were diluted in ethanol. (**A**) Acetone, (**B**) dimethyl sulfide, (**C**) 2-butanone, (**D**) ethyl acetate, and (**E**) dimethyl disulfide. Gray bars indicate concentration of VOCs used in odor bouquet at the approximate concentrations in *S. marcescens* headspace (5 mM acetone, 0.5 mM dimethyl sulfide, 0.5 mM 2-butanone, 0.1 mM ethyl acetate and 1 mM dimethyl disulfide). a indicates $P < 0.01$, one sample *t*-test compared to 0; $n \geq 6$ assays. Error bars represent SEM. (**F**) Chemotaxis to odor mixtures as labeled; concentrations are those found in *S. marcescens* headspace (5 mM acetone, 0.5 mM dimethyl sulfide, 0.5 mM 2-butanone, 0.1 mM ethyl acetate, and 1 mM dimethyl disulfide). ***$P < 0.001$, *$P < 0.05$, ANOVA with Dunnett compared to bouquet with all 5 odorants, $n \geq 6$ assays. Error bars represent SEM.

1999) resembled wild-type animals in their preference for *S. marcescens* in the choice assay. The double mutant *osm-9 ocr-2*, which lacks sensory function of ASH, ADL and AWA neurons (Tobin et al. 2002) also showed a strong preference for *S. marcescens*. However, the mutant *ceh-36*, which has a defect in AWC cell fate (Lanjuin et al. 2003; Koga and Ohshima 2004) had a reversed olfactory preference, preferring *E. coli* to *S. marcescens*. The mutant *ceh-36* also affects the differentiation of ASE taste neurons (Chang et al. 2003; Koga and Ohshima 2004), but the *che-1* mutant, which also lacks ASE neurons, retained the normal preference for *S. marcescens*. These results suggest that AWC neurons are important for *S. marcescens* preference.

We also tested the role of BAG neurons because these neurons, which respond to carbon dioxide, have been shown to be important for *C. elegans* to leave the lawn of *S. marcescens* after several hours (Brandt and Ringstad 2015). To ask whether the BAG neurons are important for the initial attraction of *C. elegans* to *S. marcescens*, we tested *gcy-9* mutants which have defective BAG neurons (Hallem et al. 2011). We found that *gcy-9* mutants showed wild-type preference for *S. marcescens* in the choice assay (Figure 4A). Thus BAG neurons are important for leaving *S. marcescens*, but not for preference for *S. marcescens* over *E. coli*.

AWC sensory transduction requires a cyclic nucleotide-gated channel (TAX-4) that is localized to the sensory cilia of multiple

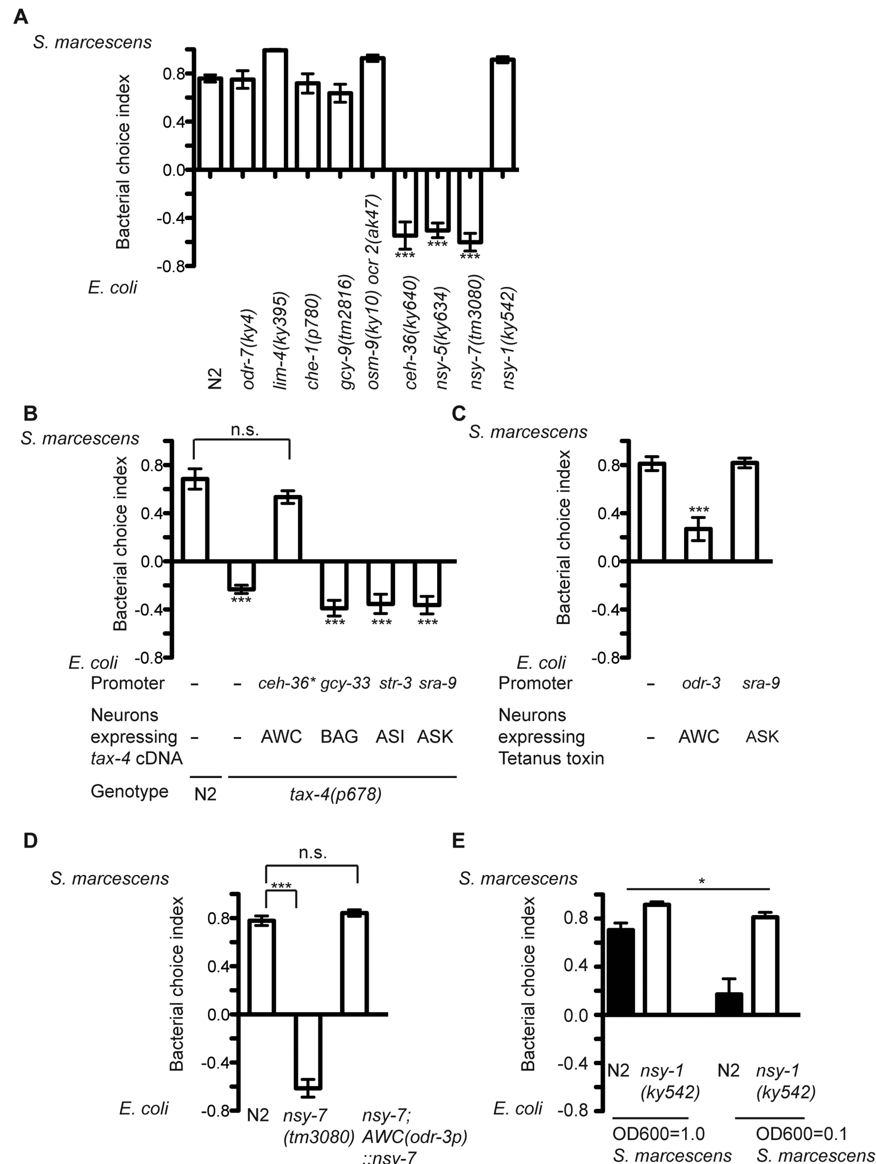

Figure 4. AWC^{ON} mediates *Caenorhabditis elegans* preference for *Serratia marcescens*. (**A**) Bacterial choice (*S. marcescens* OD600 = 10; *E. coli* OD600 = 1.0) index of mutants affecting olfactory neuron cell fates or function. (**B**) Rescue of bacterial choice behavior in *tax-4(p678)* mutants expressing *tax-4* cDNA in AWC (*ceh-36** promoter) but not BAG (*gcy-33* promoter), ASI (*str-3* promoter), or ASK (*sra-9* promoter). (**C**) Bacterial choice behavior of N2 worms expressing tetanus toxin in AWC and AWB weakly (*odr-3* promoter) or ASK (*sra-9* promoter). (**D**) *nsy-7* rescued in AWC and AWB (*odr-3* promoter). ***$P < 0.001$, compared to WT by ANOVA with Dunnett, $n \geq 6$ assays. (**E**) Effect of different concentrations of *S. marcescens* (OD600 = 1.0 or OD600 = 0.1) on bacterial choice index of WT and *nsy-1* mutant (2 AWC^{ON} neurons); the concentration of *E. coli* (OD600 = 10) was held constant. *P* value was generated by 2-way ANOVA for interaction of genotype and *S. marcescens* concentration (OD600 = 1.0 or 0.1); *P <0.05, $n \geq 5$ assays.

chemosensory neurons including AWC (Komatsu et al. 1996; Chang et al. 2003). The *tax-4* mutants were defective in *S. marcescens* preference, consistent with a role for AWC chemosensation in the choice (Figure 4A). *Serratia marcescens* preference was restored by *tax-4* expression under the *ceh-36** promoter, which is selective for AWC neurons (Chang et al. 2003; Koga and Ohshima 2004), but was not rescued by *tax-4* expression *gcy-33* (BAG) promoter, consistent with the *gcy-9* mutant data, or under a control *sra-9* (ASK) promoter (Figure 4B). *Serratia marcescens* preference was also disrupted by a tetanus toxin transgene that blocked synaptic release from AWC and AWB neurons, but not by a control ASK tetanus toxin transgene (Figure 4C). Thus the strong preference for *S. marcescens* likely requires the sensory function and synaptic output of AWC.

The AWC neuron pair consists of 2 cells, AWC^{ON} and AWC^{OFF}, that express different chemoreceptor genes and detect partly overlapping sets of odors (Troemel et al. 1999; Wes and Bargmann 2001). Animals mutant for the genes *nsy-5* and *nsy-7* have cell fate transformations that eliminate the AWC^{ON} neuron, resulting in 2 AWC^{OFF} neurons (Chuang et al. 2007; Lesch et al. 2009). Like *ceh-36* mutants, *nsy-5* and *nsy-7* were reversed in choice behaviors, preferring *E. coli* to *S. marcescens* (Figure 4A). Expression of *nsy-7* in AWC under the *odr-3* promoter fully rescued *S. marcescens* preference in a *nsy-7* background (Figure 4D), as well as rescuing the AWC^{ON} cell fate (Lesch et al. 2009). By contrast, the *nsy-1* mutant, which lacks AWC^{OFF} neurons (Sagasti et al. 2001), retained a strong preference for *S. marcescens* (Figure 4A). The effects of *nsy-5*, *nsy-7*, and *nsy-1* indicate that AWC^{ON} drives *S. marcescens* preference.

The cell fate transformation in *nsy-1* results in 2 AWC^{ON} neurons, and in a standard choice assay *nsy-1* appeared to have an even stronger preference for *S. marcescens* than wild type (Figure 4A). To confirm this result, we presented a 10-fold lower amount of *S. marcescens* bacteria in the choice with *E. coli*, a configuration that greatly diminished the wild-type preference for *S. marcescens* over *E. coli* (Figure 4E). In this sensitized choice assay, *nsy-1* mutants had a strongly enhanced preference for *S. marcescens* (Figure 4E). These results indicate that the number of AWC^{ON} neurons can determine *S. marcescens* preference: animals with 2 AWC^{ON} neurons have an even stronger preference for *S. marcescens* than wild-type animals with one AWC^{ON} neuron.

Taken together, *C. elegans* had a strong preference for the odor of *S. marcescens* over the odor of *E. coli* in a bacterial choice assay. *Serratia marcescens* preference is highly dependent on a specific class of olfactory neuron, AWC^{ON}, which is known to sense a variety of attractive volatile odors. Mutants selectively lacking AWC^{ON} or both AWC neurons had a reversed preference favoring *E. coli*, suggesting that in the absence of AWC neurons, non-AWC neurons can drive either attraction toward *E. coli* or repulsion from *S. marcescens* odors.

AWC^{ON} detects *S. marcescens* volatiles: 2-butanone and acetone

Does the AWC^{ON} neuron which mediates the olfactory preference for *S. marcescens* over *E. coli* also mediate the attraction to acetone and 2-butanone? The AWC^{ON} neuron can respond to more than one odorant because a single sensory neuron in *C. elegans* expresses many different kinds of olfactory G-protein coupled receptors (Bargmann 2006). The volatile odorant 2-butanone has previously been shown to be sensed by the AWC^{ON} neuron, but the neuron responding to acetone has not been previously determined (Bargmann 2006; Tsunozaki et al. 2008). We found that acetone was likely also sensed by the AWC^{ON} neuron because genetic mutants that affect AWC^{ON} function (*ceh-36* and *nsy-7*) fail to chemotax towards acetone (Figure 5A). In addition, *tax-4* mutants were defective in acetone chemotaxis, but wildtype acetone chemotaxis was restored by *tax-4* expression under the *ceh-36** promoter, which is selective for AWC neurons (Figure 5B).

To test whether 2-butanone and acetone can drive food choice, we conducted a modified bacterial choice assay. *Escherichia coli* was spotted on both sides and the plate and then immediately before the assay acetone (10 mM) and 2-butanone (1 mM) were added to *E. coli* patch on one side and the diluent ethanol was added to the *E. coli* patch on other side. We found that wild-type N2 animals preferred the *E. coli* patch with acetone and 2-butanone at these concentrations, 2-fold higher than the approximate concentrations in the *S. marcescens* headspace, but not at lower concentrations (Figure 5C, data not shown). We then tested the role of the AWC^{ON} neuron in detecting 2-butanone and acetone in the presence of *E. coli*. First, we examined *nsy-7* mutants in the modified 2-butanone and acetone *E. coli* choice assay. As expected, the *nsy-7* mutants which lack the AWC^{ON} neuron showed no preference for the *E. coli* patch with 2-butanone and acetone over *E. coli* alone (Figure 5C). Second, we tested *tax-4* mutants and found that they were defective in the *E. coli* plus 2-butanone and acetone assay (Figure 5D). Preference for *E. coli* with 2-butanone and acetone was restored by *tax-4* expression in AWC neurons. These results indicate that the AWC^{ON} neuron drives preference for acetone and 2-butanone in the presence of background volatiles released by *E. coli*.

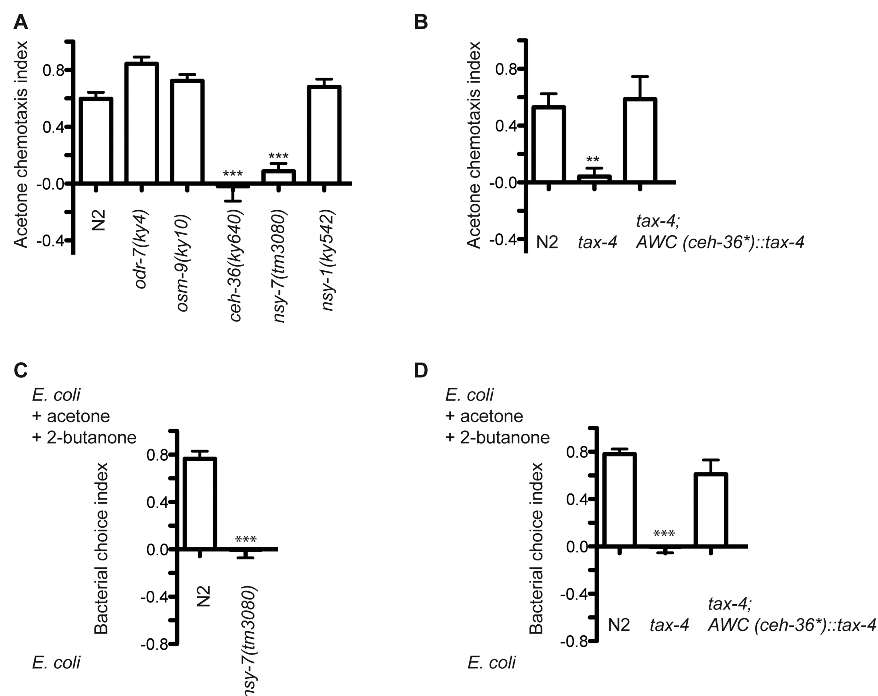

Figure 5. AWC^{ON} mediates chemotaxis to acetone and *Escherichia coli* with added acetone and 2-butanone. (**A**) Acetone chemotaxis behavior of genetic mutants affecting olfactory neuron cell fates. (**B**) Rescue of acetone chemotaxis in *tax-4(p678)* mutants expressing *tax-4* cDNA in AWC (*ceh-36** promoter). (**C**) Bacterial choice between patch of *E. coli* (OD600 = 10) with 10 mM acetone and 1 mM 2-butanone diluted in ethanol and patch of *E. coli* (OD600 = 10) with diluent ethanol. N2 shows preference for *E. coli* with acetone and 2-butanone and *nsy-7* mutant shows no preference. (**D**) Rescue of *E. coli* (OD600 = 10) with 2-butanone and acetone versus *E. coli* (OD600=10) alone in *tax-4(p678)* mutants expressing *tax-4* cDNA in AWC (*ceh-36** promoter) ***$P < 0.001$, **$P < 0.01$ compared to WT by ANOVA with Dunnett, $n \geq 6$ assays. Error bars represent SEM.

Conditioning with *S. marcescens* odor

Our results indicate that the detection of 2-butanone and acetone by the AWC^{ON} neuron can drive preference for *S. marcescens*. However, how does *C. elegans* recognize the odor of *S. marcescens*? In other words, does the 5 component odor blend, 2-component odor blend of acetone and 2-butanone, or a single odorant represent *S. marcescens* to *C. elegans*? To answer this question, we conducted a modified version of an aversive conditioning assay (Bargmann et al. 1993; Bargmann and Colbert 1995). In aversive conditioning assays, worms are exposed to an odorant in the absence of food for 90 min. These "conditioned" worms show a lower preference for that specific odorant than "naïve" worms that were starved in the absence of the odorant. We then assayed their chemotaxis to the natural odor of *S. marcescens*, single odorants present in the *S. marcescens* headspace, and blends of these odorants (Figure 6A). We hypothesized that *C. elegans* would reduce its preference for the volatile odorants that resemble its representation of the odor of *S. marcescens*. We tested conditioned worms for chemotaxis towards the natural odor of *S. marcescens* bacteria and found that *C. elegans* showed reduced chemotaxis towards the natural odor. We then tested pure odor components in the headspace of *S. marcescens*. We tested chemotaxis towards 1 mM acetone, a concentration approximately 5-fold more dilute than in the *S. marcescens* headspace because in previous pure odor conditioning experiments, worms showed a reduction in preference for odors more dilute than the conditioning odor (Bargmann and Colbert 1995). We found that *C. elegans* conditioned with *S. marcescens* reduced its chemotaxis towards acetone. This reduction in chemotaxis was similar to the reduction in chemotaxis towards the natural odor of *S. marcescens* (Figure 6B). For comparison, we tested the other components at 1 mM. We found a reduction in preference for the attractive odorant, 2-butanone, but not for neutral odorants, ethyl acetate and dimethyl disulfide (Figure 6B). As expected, the conditioned worms did not show a reduction in chemotaxis towards isoamyl alcohol, a known attractive odor detected by AWC, but not present in the headspace of *S. marcescens* (Bargmann et al. 1993).

We next tried odor mixtures. We tested the conditioned worms for chemotaxis towards the bouquet of all 5 odorants at a 5-fold lower concentration for all odorants than the approximate concentration of odorants in *S. marcescens* headspace. The conditioned worms showed a similar reduction in chemotaxis to the bouquet of all 5 odorants, the mixture of acetone and 2-butanone and the natural odor of *S. marcescens*. In addition, we found that the reduction in preference for acetone alone and for the mixture of acetone and 2-butanone were not significantly different (2-way ANOVA, $P = 0.51$). This likely indicates that the change in response to these odors is non-additive. Thus taken together, it seems the 2 components, acetone and 2-butanone, either alone or together, represent the more complex mixture of at least 5 volatiles in *S. marcescens* headspace.

Next we did the reciprocal experiment. We odor conditioned worms with different single odorants found in *S. marcescens* headspace paired with food deprivation for 90 min and then tested the conditioned and naïve worms in a *S. marcescens* and *E. coli* bacterial choice assay (Figure 7A). We hypothesized that conditioning with attractive odorants in the *S. marcescens* headspace would reduce preference for *S. marcescens*, but conditioning with neutral odorants would not. As expected, conditioning with acetone alone reduced *S. marcescens* preference greatly and conditioning with 2-butanone also reduced *S. marcescens* greatly (Figure 7B). In addition, conditioning to neutral odorants in the headspace of *S. marcescens*,

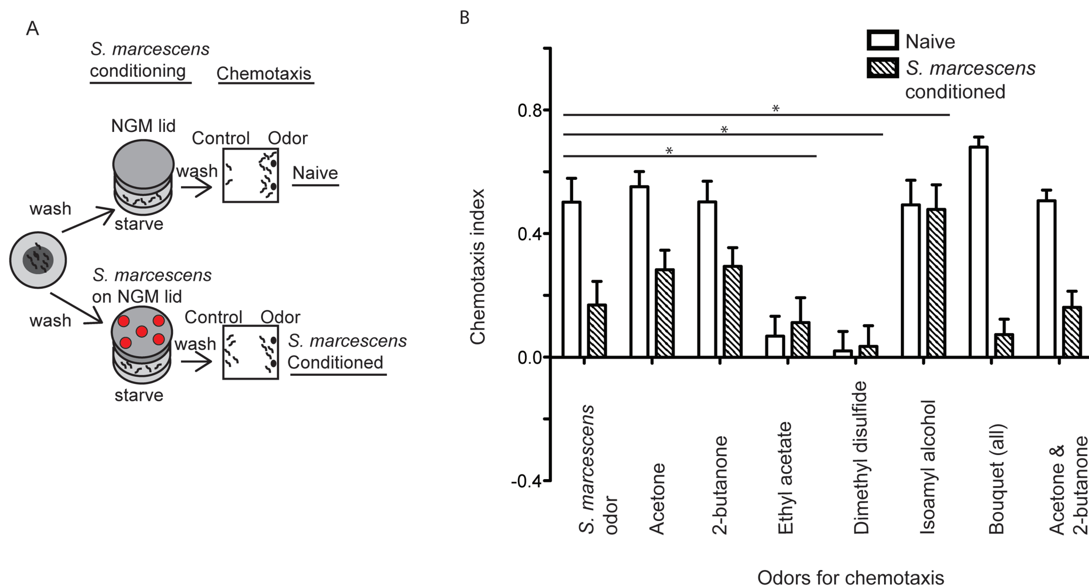

Figure 6. Aversive olfactory conditioning with odor of *Serratia marcescens* reduces preference to 2-butanone and acetone. (**A**) Schematic of aversive bacterial conditioning assay. Adult worms are washed off food, starved for 90 min in the presence of odor of *S. marcescens* (OD600 = 10) growing on NGM agar lid (*S. marcescens* conditioned) or NGM agar lid alone (naïve) and then washed again and tested in chemotaxis assays to various single odorants and odor mixtures. (**B**) Naïve and conditioned worms were tested for chemotaxis to natural odor of *S. marcescens* (bacterial patch (OD600 = 1.0) versus LB media on NGM agar on lid of chemotaxis plate); single odorant components (1 mM) present in *S. marcescens* headspace; to a mixture of all 5 odorants at 5-fold lower concentrations than approximate concentrations in *S. marcescens* headspace (1 mM acetone, 0.1 mM dimethyl sulfide, 0.1 mM 2-butanone, 0.02 mM ethyl acetate, and 0.2 mM dimethyl disulfide); a mixture of 1 mM acetone and 0.1 mM 2-butanone; and 10 mM isoamyl alcohol, an odorant not found in *S. marcescens* headspace. *P* values were generated by 2-way ANOVA for interaction of tested chemotaxis odor and condition (naïve or *S. marcescens* conditioned); each tested chemical odor or mixture of odors was compared to natural *S. marcescens* odor; as well as additional comparison between acetone alone and mixture of acetone and 2-butanone (ns, $P = 0.51$). $^*P < 0.05$, $n \geq 5$ assays. Error bars represent SEM.

ethyl acetate and dimethyl disulfide, did not reduce *S. marcescens* preference.

Last, we preexposed worms to isoamyl alcohol, which is not present in *S. marcescens* headspace, and tested *S. marcescens* preference in the bacterial choice assay. As expected, the worms did not show a reduced preference for *S. marcescens*. Next, we investigated the response of the worms after being conditioned with an odor blend. We conditioned food deprived worms with a mixture of acetone (present in *S. marcescens*) and isoamyl alcohol (absent in *S. marcescens*) and then assayed preference. We hypothesized that worms would not lower their preference for *S. marcescens* because this blend of acetone and isoamyl alcohol would be very different from the odor of *S. marcescens*. However, they did reduce their preference. Thus, the worms seem to be attending to the components of the conditioning odor blend and so now avoid an odor blend that contains acetone, but is otherwise very different from the conditioning odor blend.

Discussion

Caenorhabditis elegans is attracted to volatile chemicals released by bacteria and can distinguish among the blends of volatiles released by different species of bacteria, which is important for finding nutritious food and avoiding harmful microbes (Bargmann et al. 1993; Zhang et al. 2005; Shtonda and Avery 2006). However, the precise odors that *C. elegans* uses to detect and recognize a specific bacterial species have not been identified. In this study, we determined that the volatile chemical cues mediating recognition of pathogenic *S. marcescens* are 2-butanone and acetone. The amphid chemosensory neuron, AWC^{ON}, senses both *S. marcescens*-released 2-butanone and acetone and is necessary for attraction to the natural odor of *S. marcescens*. In an aversive learning assay where the odor of *S. marcescens* is paired with food deprivation, *C. elegans* reduces its chemotaxis similarly for the blend of acetone and 2-butanone and the odor of *S. marcescens*, indicating that the blend of acetone and 2-butanone represents *S. marcescens* to *C. elegans*.

The neuronal basis of preference for *S. marcescens*

The standard *C. elegans* laboratory strain N2 has a strong preference for the odor of *S. marcescens* over the odor of *E. coli HB101* in an olfactory choice assay. *S. marcescens* preference is highly dependent on a specific class of olfactory neuron, AWC^{ON}, which is known to sense a variety of attractive volatile odors. Interestingly, AWC^{ON} has been shown to recognize several ketones, including 2-butanone, 2-heptanone and in this study, acetone (Bargmann et al. 1993; Zhang et al. 2016). Mutants selectively lacking AWC^{ON} or both AWC neurons had a reversed preference favoring *E. coli*, suggesting that non-AWC neurons can drive either attraction toward *E. coli* or repulsion from *S. marcescens* odors.

In a previous study, N2 preference for the bacterium *Pseudomonas aeruginosa PA14* over *E. coli OP50* was found to require both AWC and AWB olfactory neurons (Ha et al. 2010). Although both *Serratia* and *Pseudomonas* preference required AWC, a complete reversal of preference was observed only in the *S. marcescens*–*E. coli* choice, and not in the *Pseudomonas*–*E. coli* choice. Moreover, in our assay the AWB mutant *lim-4* did not affect *S. marcescens* preference. These results suggest that the identity of the neurons that mediate olfactory choice behavior and the magnitude of their effects vary based on the specific bacteria that are tested.

Volatiles in *S. marcescens* headspace

We identified 5 major volatiles in the *S. marcescens* headspace, or volume of air above bacteria within the GC-MS vial. We would like to highlight that there may be additional low abundance volatiles in the *S. marcescens* headspace that were not detected by the methods used in this study. We intentionally grew the bacteria for GC-MS analysis in as similar a way as possible to the way the bacteria were grown in the bacterial choice assay. In particular, this meant that the bacteria were grown on NGM agar for 5 h and then placed in a GC-MS glass vial for only 1 h because that because this means that the bacteria is in the same bacterial growth state as during 1 h bacterial choice assays (Supplementary Figure 1). However, this short incubation time in the GC-MS glass vial means that high abundance volatiles are detected, but low abundance volatiles may be under the

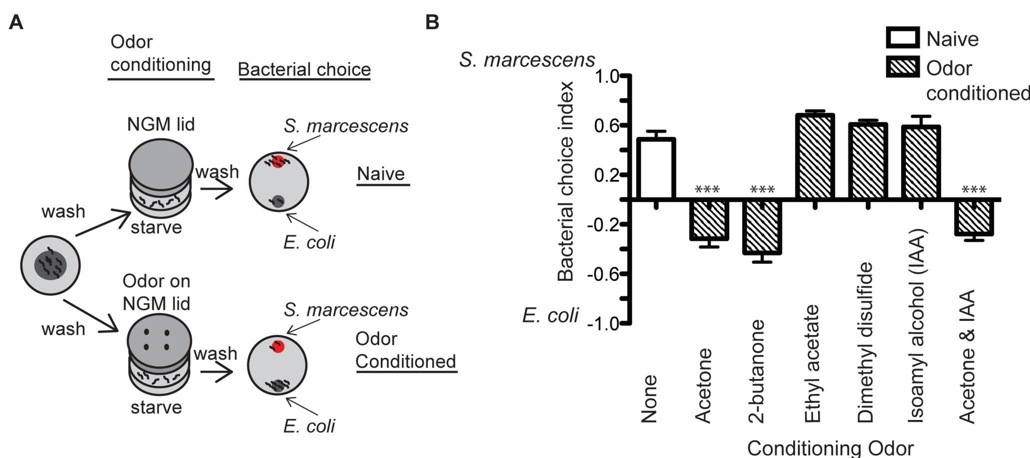

Figure 7. Aversive olfactory conditioning with single odorants reduces preference for *Serratia marcescens*. (**A**) Schematic of aversive odor conditioning assay. Adult worms are washed off food, starved for 90 min in the presence of odor on NGM agar lid (odor conditioned) or NGM agar lid alone (naïve) and then washed again and tested in bacterial choice assays between *S. marcescens* (OD600 = 1.0) and *E. coli* (OD600 = 10). (**B**) Odor conditioned worms were exposed to single odor components present in *S. marcescens* headspace (acetone, 2-butanone, ethyl acetate, and dimethyl disulfide) as well as a single control odorant not found in *S. marcescens* headspace (isoamyl alcohol). In addition, worms were conditioned to a mixture of an odorant present in *S. marcescens* headspace, acetone, and absent, isoamyl alcohol (IAA). ***$P < 0.001$, odor conditioned worms compared to naïve worms by ANOVA with Dunnett, $n \geq 6$ assays. Error bars represent SEM.

threshold for detection. Nevertheless, we found that the high abundance volatiles that we did detect, acetone and 2-butanone, were sufficient to represent *S. marcescens* in aversive olfactory conditioning assays and to drive behavior.

To determine, the similarity of odor profiles of different strains of *S. marcescens*, we examined 2 additional strains of *S. marcescens*, pathogenic Db11 and its non-pathogenic derivative Db1140 (Kurz et al. 2003). Db11 and Db1140 are non-pigmented strains of *S. marcescens* that do not produce the red pigment prodigiosin (Thomson et al. 2000). Non-pigmented *S. marcescens* strains are more commonly found in hospitals and involved in human infections while pigmented strains, such as ATCC 274 used in this study, are more commonly found in natural environments (Hejazi and Falkiner 1997). We found that Db11 released volatiles similar to *S. marcescens* ATCC 274: acetone, 2-butanone and dimethyl disulfide, but not ethyl acetate or dimethyl sulfide (Supplementary Table 1 and Figure 2). Db11 also released 2-pentatone, which was not detected in the headspace of ATCC 274. We found that the headspace of non-pathogenic derivative Db1140 differed somewhat from pathogenic Db11. Db1140-released acetone and pyrrole, which was not detected in Db11 or ATCC 274, but not 2-butanone and dimethyl disulfide (Supplementary Table 1 and Figure 2). Interestingly, if Db1140 was grown over a longer period of time (5 h instead of 1 h) in the GC-MS glass vial from which the headspace was sampled, 2-butanone was detected, but not dimethyl disulfide (data not shown). This result likely means that Db1140 does release 2-butanone, but at much lower levels than Db11. Thus, the 3 *S. marcescens* strains examined are similar in that they all release the attractive odorants acetone and 2-butanone, but there are differences among the odor profiles of the strains as well.

Recognition of odor blends by *C. elegans*

We examined how *C. elegans* recognizes an odor blend. We found that although we detected 5 major volatiles in the headspace of *S. marcescens*, *C. elegans* was only attracted to 2 out of these 5 volatiles, acetone and 2-butanone. We next asked what component or components of the odor blend of *S. marcescens* results in reduced chemotaxis after pairing the odor of *S. marcescens* with food deprivation. We found that acetone and 2-butanone represent *S. marcescens* to *C. elegans*. This result is similar to insects, which in the majority of studies, use a few key components to recognize an odor blend of volatiles from host plants (Pham-Delegue et al. 1993; Bruce et al. 2005). Although *C. elegans* is estimated to have approximately 2000 olfactory receptors and may have the olfactory repertoire to respond to many components in a mixture, at least in this case, it only responded behaviorally to 2 representative components in olfactory association assays.

It is noteworthy that 2-butanone is one of the odorants for recognition of *S. marcescens* because 2-butanone is a commonly used odorant to study olfactory plasticity in the *C. elegans* literature. For example, when 2-butanone is paired with food deprivation, *C. elegans* decreases its attraction to 2-butanone (Bargmann and Colbert 1995). In addition, when 2-butanone is paired with feeding, *C. elegans* increases its attraction to 2-butanone (Torayama et al. 2007). Thus, this study shows that 2-butanone is likely an ethologically relevant stimulus to *C. elegans*.

These odorants, 2-butanone and acetone, are not unique to *S. marcescens*. In fact, they are ubiquitous and released by many species of bacteria, both pathogenic and non-pathogenic (Lemfack et al. 2014). We propose that because many species of bacteria release similar volatiles, *C. elegans* must distinguish among them by recognizing different ratios of the attractive components of the odor mixture. Indeed, insects have been shown mostly to recognize the volatiles of host plants by different ratios of constitutive components as opposed to components that are unique to specific host plants (Bruce and Pickett 2011). In order to test this hypothesis, the components and ratio of components needed for recognition of bacteria by *C. elegans* will need to be determined for several species of bacteria. We are currently working on this for bacterial species isolated from the natural microbiome of *C. elegans* (Samuel et al. 2016).

Attraction to pathogenic *S. marcescens*

It is surprising that *C. elegans* is strongly attracted to the odor of *S. marcescens*, a pathogenic bacterium that can kill infected animals in a few days. Interestingly, *C. elegans* is also attracted to the odors of other harmful microbes. In 2 recent studies, pathogenic bacterium *Bacillus nematocida* and the nematophagous fungus *Arthrobotrys oligospora* were shown to be attractive to *C. elegans* and to release ubiquitous volatiles as well (Niu et al. 2010; Hsueh et al. 2017). Although there should be a strong selection for avoidance of odors of harmful microbes, if harmful and harmless microbes release similar volatiles, how can *C. elegans* avoid them? On a short timescale, *C. elegans* can learn to avoid microbes that cause an adverse experience (Zhang et al. 2005). Over multiple generations, natural populations of *C. elegans* may evolve to avoid the microbes in its local environment. Indeed, in our previous work we found that level of attraction for the odor of *S. marcescens* among wild strains of *C. elegans* does vary, which indicates that the level of attraction may indeed be a rapidly evolving trait (Glater et al. 2014). In addition in an experimental co-evolution study after being passaged with the *S. marcescens* for 30 generations, *C. elegans* was shown to increase its avoidance of the pathogen, based on both olfactory and non-olfactory cues (Penley and Morran 2017). Thus, the ability to modify preferences for specific microbes within an organism's lifetime as well as on an evolutionary timescale may be an important feature of how *C. elegans* navigates the complex microbial environment.

In conclusion, we have determined that 2 key components represent the natural blend of *S. marcescens* to *C. elegans*. This finding is both an important step forward for understanding the chemical basis of the interaction between *C. elegans* and *S. marcescens* as well as the first study to examine how the worm recognizes odor blends. Knowledge about how *C. elegans* recognizes natural odor blends is likely to be useful for increased understanding of how evolutionarily related parasitic nematodes recognize their human, animal or plant hosts, and for development of reagents that could be useful for nematode control. We expect that this work will provide the foundation for future work to elucidate further the chemical, neuronal, and molecular basis of microbe recognition by *C. elegans* and their role in host-pathogen coevolution.

Supplementary material

Supplementary material are available at *Chemical Senses* online.

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