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ENVIRONMENTAL MICROBIOLOGY

The Ubiquitous Soil Terpene Geosmin Acts as a Warning Chemical

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ABSTRACT Known as the smell of earth after rain, geosmin is an odorous terpene detectable by humans at picomolar concentrations. Geosmin production is heavily conserved in actinobacteria, myxobacteria, cyanobacteria, and some fungi, but its biological activity is poorly understood. We theorized that geosmin was an aposematic signal used to indicate the unpalatability of toxin-producing microbes, discouraging predation by eukaryotes. Consistent with this hypothesis, we found that geosmin altered the behavior of the bacteriophagous nematode *Caenorhabditis elegans* on agar plates in the absence of bacteria. Normal movement was restored in mutant worms lacking differentiated ASE (amphid neurons, single ciliated endings) neurons, suggesting that geosmin is a taste detected by the nematodal gustatory system. In a predation assay, geosmin and the related terpene 2-methylisoborneol reduced grazing on the bacterium *Streptomyces coelicolor*. Predation was restored by the removal of both terpene biosynthetic pathways or the introduction of *C. elegans* that lacked differentiated ASE taste neurons, leading to the apparent death of both bacteria and worms. While geosmin and 2-methylisoborneol appeared to be nontoxic, grazing triggered bacterial sporulation and the production of actinorhodin, a pigment coproduced with a number of toxic metabolites. In this system, geosmin thus appears to act as a warning signal indicating the unpalatability of its producers and reducing predation in a manner that benefits predator and prey. This suggests that molecular signaling may affect microbial predator-prey interactions in a manner similar to that of the well-studied visual markers of poisonous animal prey.

IMPORTANCE One of the key chemicals that give soil its earthy aroma, geosmin is a frequent water contaminant produced by a range of unrelated microbes. Many animals, including humans, are able to detect geosmin at minute concentrations, but the benefit that this compound provides to its producing organisms is poorly understood. We found that geosmin repelled the bacterial predator *Caenorhabditis elegans* in the absence of bacteria and reduced contact between the worms and the geosmin-producing bacterium *Streptomyces coelicolor* in a predation assay. While geosmin itself appears to be nontoxic to *C. elegans*, these bacteria make a wide range of toxic metabolites, and grazing on them harmed the worms. In this system, geosmin thus appears to indicate unpalatable bacteria, reducing predation and benefiting both predator and prey. Aposematic signals are well known in animals, and this work suggests that metabolites may play a similar role in the microbial world.

KEYWORDS 2-methylisoborneol, geosmin, aposematism, *Caenorhabditis elegans*, chemical ecology, natural products, predation, prey, warning chemical, warning signal

Few natural products are as widespread as geosmin, the smell of wet earth (1). Its chief producers in the soil, the saprophytic bacterial phylum *Actinobacteria* and the predatory/saprophytic bacterial order *Myxococcales*, are found on every continent, including Antarctica (2, 3). Geosmin is also a common contaminant in drinking water and farmed fish (4, 5), produced by aquatic equivalents of these soil bacteria and by

Editor Hideaki Nojiri, University of Tokyo

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The authors declare no conflict of interest.

Received 18 January 2022
Accepted 26 February 2022

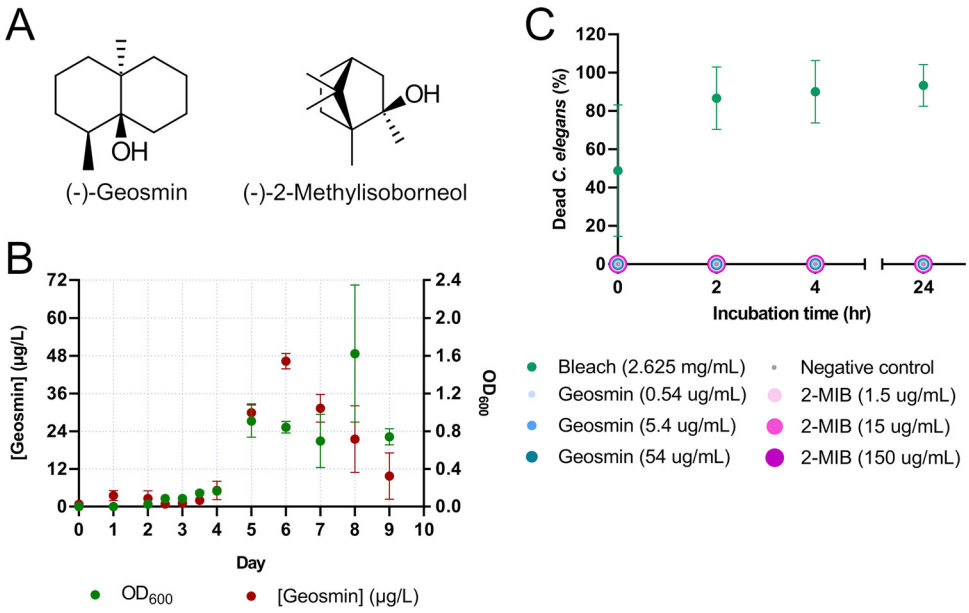

FIG 1 Geosmin production and toxicity. (A) Chemical structures of geosmin and 2-methylisoborneol. (B) Growth curve and geosmin production of *M. xanthus* DK1622 in 1% CTT. Geosmin concentrations were determined via GC-MS, with the aid of a calibration curve derived from solutions of authentic geosmin. Cells began to clump at day 7, decreasing the reliability of OD_{600} measurements. (C) Viability of adult *C. elegans* hermaphrodites in the presence of geosmin, 2-methylisoborneol (2-MIB), and bleach. Worms were coded as nonresponsive if they did not move following touch stimuli. Experiments were conducted in triplicate with five adult *C. elegans* hermaphrodites per well ($n = 15$).

aquatic and terrestrial cyanobacteria (4, 6). Although chiefly a bacterial metabolite, geosmin is also produced by a range of fungi (7, 8) and is found in the peel of beets (9). Geosmin production is heavily conserved (6, 10–12) and frequently accompanied by another odorous terpene, 2-methylisoborneol (Fig. 1A) (11, 13).

The breadth of geosmin producers and their varied ecological niches complicate the assignment of the compound's broader function. Geosmin repels egg-laying *Drosophila melanogaster*, which has a dedicated olfactory sensory neuron for geosmin detection (14), and attracts mosquitos and ants (9, 15). Geosmin attracts springtails to sporulating *Streptomyces* colonies, which then eat the bacteria and help disperse bacterial spores (16), but this interaction would be of limited utility to the aquatic organisms that produce geosmin (6). Due to differing primary nutrient sources, life cycles, cell wall structures, predators, and symbionts, the principal geosmin producers have few features in common (6, 10, 11), but the three main clades (actinobacteria, myxobacteria, and cyanobacteria) all produce a wealth of nongeosmin secondary metabolites (17). Many of these bioactive compounds inhibit the growth of bacteria or eukaryotes (17, 18) and may be used in nature to deter competitors and predators (19–22).

In the animal kingdom, toxic prey advertise their unpalatability through the use of warning colors (23). These bright colors make the prey more conspicuous, but when combined with negative stimuli, they deter predation through learned responses (24, 25). To date, no warning colors or other aposematic signals have been identified in bacteria, although olfactory signals may be used to reduce the scavenging of nutrient-rich insects killed by entomopathogenic bacteria (26).

We propose that geosmin may act as a warning chemical, advertising the production of toxic secondary metabolites. Commensurate with this hypothesis, we found that while geosmin was nontoxic to the bacteriophagous nematode *Caenorhabditis elegans*, it triggered a worm movement with both the rapid speed of roaming movement and the frequent changes in direction common to dwelling movement. This change in movement behavior was absent in worms lacking functional ASE (amphid neurons, single ciliated endings) neurons. Geosmin acted as a repellent in a subsequent contact-mediated aversion assay but had no visible effect on worm viability. The addition of geosmin or the related terpene 2-methylisoborneol to

Streptomyces coelicolor but not *Escherichia coli* reduced grazing in a predation assay, while grazing on *S. coelicolor* or the alternate geosmin producer *Myxococcus xanthus* led to stress behaviors and the death of the worms. The use of geosmin as a warning chemical targeted to *C. elegans* is in line with previous reports of its attractant and repellant activities and provides an explanation for the high prevalence of geosmin biosynthetic genes in evolutionarily disparate microbes well known for their toxic natural products.

RESULTS

Geosmin is produced during growth. To determine when geosmin provides the most benefit to its producers, we characterized the production of geosmin as a function of growth in the predatory bacterium *M. xanthus* DK1622. In liquid medium, geosmin concentrations rose during the bacterium's exponential phase and reached a peak during early stationary phase, decreasing thereafter (Fig. 1B). Fractionation of the cells and medium and subsequent extraction with ethyl acetate (EtOAc) confirmed that the majority of geosmin was in the culture medium (see Table S1 in the supplemental material). This production of geosmin during exponential phase suggests that the compound provides a fitness benefit when *M. xanthus* is hunting other bacteria.

Geosmin does not aid in protein degradation. Myxobacteria and actinobacteria obtain nutrients through the degradation of organic matter, as either saprophytes or predators (2, 27). After confirming that geosmin did not inhibit the growth of Gram-negative or Gram-positive bacteria at physiologically relevant concentrations (Table S2), we examined the effect of geosmin on protease activity to determine if its biological role could be to improve nutrient accessibility. Geosmin slightly reduced the degradation of fluorescein thiocarbamoyl-casein (FTC-casein) by the digestive enzymes excreted by *M. xanthus* DK1622 (28) and had no effect on degradation by a well-characterized human protease, trypsin (Fig. S1). Given the small scale of this effect, it seems unlikely that aiding in protein degradation is geosmin's physiological role.

As geosmin associates with membrane proteins in cyanobacteria (4), we also postulated that it might stabilize hydrophobic digestive enzymes in *M. xanthus*. To evaluate this function, we measured the effect of geosmin on heat-induced denaturation of the model hydrophobic protein bovine serum albumin (BSA). Geosmin had no discernible effect on BSA stability as measured by circular dichroism (CD) spectroscopy, either at elevated temperatures or upon heating or cooling (Fig. S2).

Geosmin alters nematode behavior. To determine if geosmin is a cue that deters bacterial predators, we tested its toxicity against the nematode *C. elegans*. In addition to their use as a model organism for the study of neuronal development and function (29), nematodes are prevalent in soils across the globe, and their grazing impacts microbial abundance and diversity (30, 31). *C. elegans* nematodes are typically maintained on nematode growth medium (NGM) agar plates with *E. coli* OP50, a bacterial strain that lacks geosmin biosynthetic genes, as a food source (29, 32). When wild-type (WT) (N2) adult hermaphrodites were placed on NGM plates containing geosmin at up to 54 μg/mL, there were no obvious changes in their health over a 24-h period (Fig. 1C; Fig. S3A). Similarly, worms continued to graze on *E. coli* OP50 in the presence of geosmin, even at high concentrations, as evidenced by the deep tracks in the *E. coli* lawn after 24 h of incubation (Fig. S3B and C).

Despite having no obvious impact on the health of *C. elegans* nematodes, geosmin strongly altered their movement. *C. elegans* typically displays roaming or dwelling movements, where roaming describes long movement with few distinct turns or reversals and dwelling refers to rapid changes in direction that cause little overall displacement (33). The time spent roaming versus dwelling can be influenced by chemosensation. We found that on NGM plates lacking bacteria, adult hermaphrodites exhibited both roaming and dwelling movements (Movie S1) (34). On NGM-geosmin plates, the nematodes appeared agitated, moving with higher velocity and making more frequent changes in direction than worms in the absence of geosmin, with increased lateral displacement inconsistent with either roaming or dwelling (Movie S2). Using the Imaris and WormLab software packages, we quantified these changes in behavior, linking the

presence of geosmin to statistically significant changes in track linearity, peristaltic speed, and head movement periodicity (Table 1; see Materials and Methods for details). Since these changes in movement behavior occurred in the absence of food, it is likely that they were caused by chemosensation.

To determine how geosmin is sensed by worms, we then determined how mutants with defective chemosensation for volatile (olfaction) or soluble (gustation) chemicals moved in the presence of geosmin. As several of these mutants displayed altered mobility relative to wild-type worms in the absence of geosmin, absolute changes in track linearity, peristaltic speed, and head movement periodicity were scored. Worms with impaired pharyngeal pumping and egg laying, CE1258 [*eat-16*(*ep273*)], continued to respond to geosmin, while those deficient in the detection of a range of volatile and soluble odorants, NL2105 [*gpa-3*(*pk35*); *odr-3*(*n1605*)], failed to respond, indicating that the terpene's effect is linked to the olfactory or gustatory system (Table 1; Movies S3 and S4) (35, 36). Worms deficient in olfaction, CX2065 [*odr-1*(*1936*)] and CX2205 [*odr-3* (*n2150*)], continued to respond to geosmin (Table 1), although CX2205 had similar track linearity when geosmin was present or absent ($P = 0.0531$). While CX2205 worms are deficient in their response to some water-soluble odorants (37), these mutants also displayed altered mobility independent of cues. Regardless, PR674 [*che-1*(*p674*)] worms, where ASEL and ASER cells failed to differentiate, did not respond to geosmin, implicating a role for these neurons in geosmin sensing. BR5514 [*tax-2*(*p671*); *tax-4* (*p678*)] mutants, worms that have an ectopic ASE fate, also displayed a loss in geosmin sensing according to some parameters, while geosmin sensing was retained in strain CX5893 {*kyIs140*[*str-2*::*GFP* + *lin-15*(+)]; *ceh-36*(*ky646*)}, which is defective in ASEL identity but not ASER (38). These data support the conclusion that *C. elegans* detects geosmin through interactions mediated by the ASER neuron.

The ASE gustatory neurons have been previously linked to the sensing of water-soluble attractants and adaptive food-leaving behavior (39, 40), and thus, to confirm that geosmin can be "tasted" by *C. elegans*, we investigated the compound's activity in a dry-drop avoidance assay and a vapor diffusion assay (Fig. 2; Table S3) (41). Worms that encountered evaporated drops of either geosmin or the positive control sodium dodecyl sulfate (SDS) on NGM agar plates reversed their movement at rates significantly above those of worms passing over evaporated droplets of water (standard deviation [s] = 0.00154 and 0.000113, respectively) (Fig. 2). Geosmin was approximately 12 times more effective on a molar basis than SDS and 80 times more effective than the previously identified *Streptomyces* repellent dodecanoate (41). When combined, SDS and geosmin were more effective than either compound alone (s = 0.00519 and 0.00982, respectively) (Fig. 2), but when combined with the chemoattractant lysine, geosmin lost all apparent chemorepellent activity (s = 0.592) (Fig. 2) (42). In line with previous reports, we found that geosmin could act as a weak chemorepellent in a vapor diffusion assay (43), although the effect was weak at 0.54 μg/mL (Table S3) (chemotaxis index of -0.117; s = 0.065). This suggests that worms were unable to efficiently detect geosmin at the larger scales used in the vapor diffusion assay. Combined, these two assays indicate that geosmin is a water-soluble repellent for *C. elegans*.

Geosmin reduces interactions between *C. elegans* and its producers. To determine the benefit that geosmin provides to its native producers, we added *C. elegans* adult hermaphrodites to plates containing colonies of *S. coelicolor*. Specifically, we registered the number of worms localized within colonies of wild-type *S. coelicolor* M145 or *S. coelicolor* mutants lacking geosmin synthase (*S. coelicolor* J3003 [Δ*geoA*]) or geosmin synthase and 2-methylisoborneol (2-MIB) synthase (*S. coelicolor* J2192 [Δ*geoA* Δ*mibAB*]) (16, 44). When N2 adult hermaphrodites were added to *S. coelicolor* M145 or J3003, significantly more worms localized outside the bacterial colonies after 4 h than when worms were added to *S. coelicolor* J2192 (Fig. 3A) (s = 0.0426 and s = 0.0494, respectively), indicating that geosmin and/or 2-MIB reduced grazing on *S. coelicolor*. PR674 worms lacking functional ASE gustatory neurons were predominantly found

TABLE 1 Effect of geosmin on *C. elegans* movement[a]

Analysis parameter	*C. elegans* strain	*C. elegans* genotype	Phenotypic variation(s)	Value for treatment				
				Control		Geosmin		
				Avg	SD	Avg	SD	*P* value
Track line (%)	N2	Wild type	—	0.4495	0.2464	0.5547	0.3115	0.0498
	BR5514	*tax-2*(*p671*); *tax-4*(*p678*)	Chemosensory response, aqueous	0.4371	0.3032	0.5704	0.3091	0.0407
	CE1258	*eat-16*(*ep273*)	Chemical response, drug, movement	0.5447	0.3098	0.3973	0.2477	0.0098
	CX2065	*odr-1*(*n1936*)	Chemosensory response, odorant	**0.5715**	**0.2969**	**0.7174**	**0.2617**	**0.0531**
	CX2205	*odr-3*(*n2150*)	Chemosensory response, odorant, movement	**0.5676**	**0.2667**	**0.5015**	**0.3330**	**0.2376**
	CX5893	*kyIs140*[*str-2*::*GFP* + *lin-15*(+)]; *ceh-36*(*ky646*)	ASEL cell fate, chemosensory response, odorant and aqueous	0.3797	0.2383	0.5124	0.3134	0.0233
	NL2105	*gpa-3*(*pk35*) *odr-3*(*n1605*)	Chemosensory response, movement	**0.5417**	**0.3210**	**0.5919**	**0.3253**	**0.4034**
	PR674	*che-1*(*p674*)	ASE cell fate, chemosensory response, aqueous	**0.5617**	**0.2997**	**0.6174**	**0.2746**	**0.3523**
Peristaltic speed (μm/s) (absolute peristaltic track length/ time)	N2	Wild type	—	30.30	8.037	40.90	5.740	1.13E−06
	BR5514	*tax-2*(*p671*); *tax-4*(*p678*)	Chemosensory response, aqueous	**28.61**	**11.43**	**28.63**	**10.74**	**0.8719**
	CE1258	*eat-16*(*ep273*)	Chemical response, drug, movement	38.48	5.186	30.76	5.138	1.55E−05
	CX2065	*odr-1*(*n1936*)	Chemosensory response, odorant	31.70	3.094	45.48	6.195	5.57E−05
	CX2205	*odr-3*(*n2150*)	Chemosensory response, odorant, movement	41.43	1.541	35.47	1.380	0.00353
	CX5893	*kyIs140* [*str-2*::*GFP* + *lin-15*(+)]; *ceh-36*(*ky646*)	ASEL cell fate, chemosensory response, odorant and aqueous	27.09	9.214	35.18	5.318	0.001547
	NL2105	*gpa-3*(*pk35*) *odr-3*(*n1605*)	Chemosensory response, movement	**24.35**	**3.870**	**26.04**	**5.000**	**0.2812**
	PR674	*che-1*(*p674*)	ASE cell fate, chemosensory response, aqueous	**52.01**	**3.823**	**47.42**	**6.402**	**0.2098**
Periodicity (μm)	N2	Wild type	—	19.10	4.489	19.49	0.4998	0.03744
	BR5514	*tax-2*(*p671*); *tax-4*(*p678*)	Chemosensory response, aqueous	**18.13**	**1.357**	**17.20**	**2.389**	**0.2657**
	CE1258	*eat-16*(*ep273*)	Chemical response, drug, movement	16.39	0.4316	12.12	1.406	2.44E−18
	CX2065	*odr-1*(*n1936*)	Chemosensory response, odorant	16.09	1.204	18.69	0.3646	1.94E−05
	CX2205	*odr-3*(*n2150*)	Chemosensory response, odorant, movement	17.04	0.2397	15.15	0.6046	2.91E−05
	CX5893	*kyIs140*[*str-2*::*GFP* + *lin-15*(+)]; *ceh-36*(*ky646*)	ASEL cell fate, chemosensory response, odorant and aqueous	**19.59**	**4.840**	**22.03**	**0.8856**	**0.09487**
	NL2105	*gpa-3*(*pk35*) *odr-3*(*n1605*)	Chemosensory response, movement	**16.50**	**2.778**	**15.42**	**0.6920**	**0.6440**
	PR674	*che-1*(*p674*)	ASE cell fate, chemosensory response, aqueous	**22.65**	**2.821**	**21.98**	**0.9865**	**0.1370**

[a]Movement of adult *C. elegans* hermaphrodites on NGM and NGM-geosmin (0.54 μg/mL) plates was quantified with Imaris (track line) and WormLab (peristaltic speed and periodicity). Experiments were conducted in triplicate with three worms per plate ($n = 9$). Boldface type indicates mutant strains where geosmin sensing was lost compared to the control, as indicated by *P* values of >0.05. Dashes indicate no phenotypic variation(s).

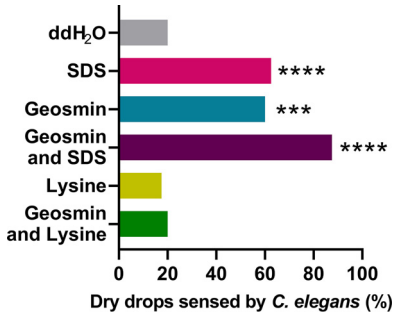

FIG 2 Dry-drop response. Proportions of adult *C. elegans* hermaphrodites that sensed 2.25 μg/mL geosmin, 43.3 μg/mL SDS, or a combination of both were determined. Animals were placed on an agar plate and allowed to move freely. A droplet of distilled, deionized water; 2.25 μg/mL geosmin; 43.3 μg/mL SDS; and/or 58.4 μg/mL lysine was placed ahead of the worms and allowed to dry (all compounds were dissolved in ddH_2O). Worms that reversed movement upon contacting the dried drop were registered as responding. *P* values were calculated via independent-sample Z tests and are indicated relative to ddH_2O (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

within colonies of all three bacterial strains at all time points (Fig. 3B). To determine if the role of the ASE neuron in bacterial avoidance was specific to *S. coelicolor*, we repeated our assay with *M. xanthus* DK1622. As with *Streptomyces*, significantly more PR674 adult hermaphrodites were found in the *M. xanthus* colony than the N2 (wild-type) nematode strain (Fig. 3C), this time at the 24-h mark.

Nematodes consumed the bacteria during all predation assays, as noted by the presence of bacteria within the nematode pharynx (Fig. 4A). Conversely, worms grazing on *M. xanthus* colonies appeared to be degraded by the digestive enzymes of these bacteria (Fig. 4B). The addition of worms to *S. coelicolor* also led to rapid sporulation and the production of actinorhodin, a pigment often coregulated with other *Streptomyces* secondary metabolites (Fig. 4C) (45). The majority of worms within *S. coelicolor* colonies at the 24-h mark were also coated in white bacterial spores and exhibited behaviors consistent with stress (Movie S5). Similar to the previously reported interactions of springtails and *S. coelicolor* (16), the movement of worms into and out of bacterial colonies did disperse some bacterial spores (Fig. 4C), but given the high toll on both bacterial growth and worm health, the overall effect of nematode predation was detrimental to both nematodes and bacteria.

To ensure that the nonterpene metabolite profiles of *S. coelicolor* mutants J3003 and J2192 did not meaningfully differ from that of the wild type, we conducted a series of add-in experiments. The addition of geosmin and/or 2-MIB to colonies of *S. coelicolor* J2192 at concentrations approximating its physiological value significantly reduced the number of worms in bacterial colonies at the 2-h, 4-h, and 24-h marks (Fig. 3D) (4, 16). When both geosmin and 2-MIB were added, the proportion of worms in the bacteria decreased over the experimental period from 44.6% at 2 h to 17.8% at 24 h ($s = 0.00912$), although it is unclear if the terpenes reduced the movement of worms into the colonies or increased colony-leaving rates. The addition of geosmin and/or 2-MIB to the standard *C. elegans* prey *E. coli* OP50 did not reduce the proportion of worms in these bacteria at any time point, indicating that the terpenes were individually necessary but not sufficient to reduce predation by *C. elegans* (Fig. 3E). Consistent with reduced exposure to toxic bacterial metabolites, digestive enzymes, and spores, geosmin addition to *S. coelicolor* J2192 reduced the proportion of worms that appeared dead at the 24-h mark from 37.2% to 10.4% ($s = 0.00430$). While the proportion of dead bacteria could not be quantified, reduced grazing by *C. elegans* provides a clear fitness benefit to the prey bacteria.

DISCUSSION

The high prevalence of geosmin synthase genes in unrelated bacterial and fungal clades suggests that geosmin is key to the fitness of a broad range of microorganisms

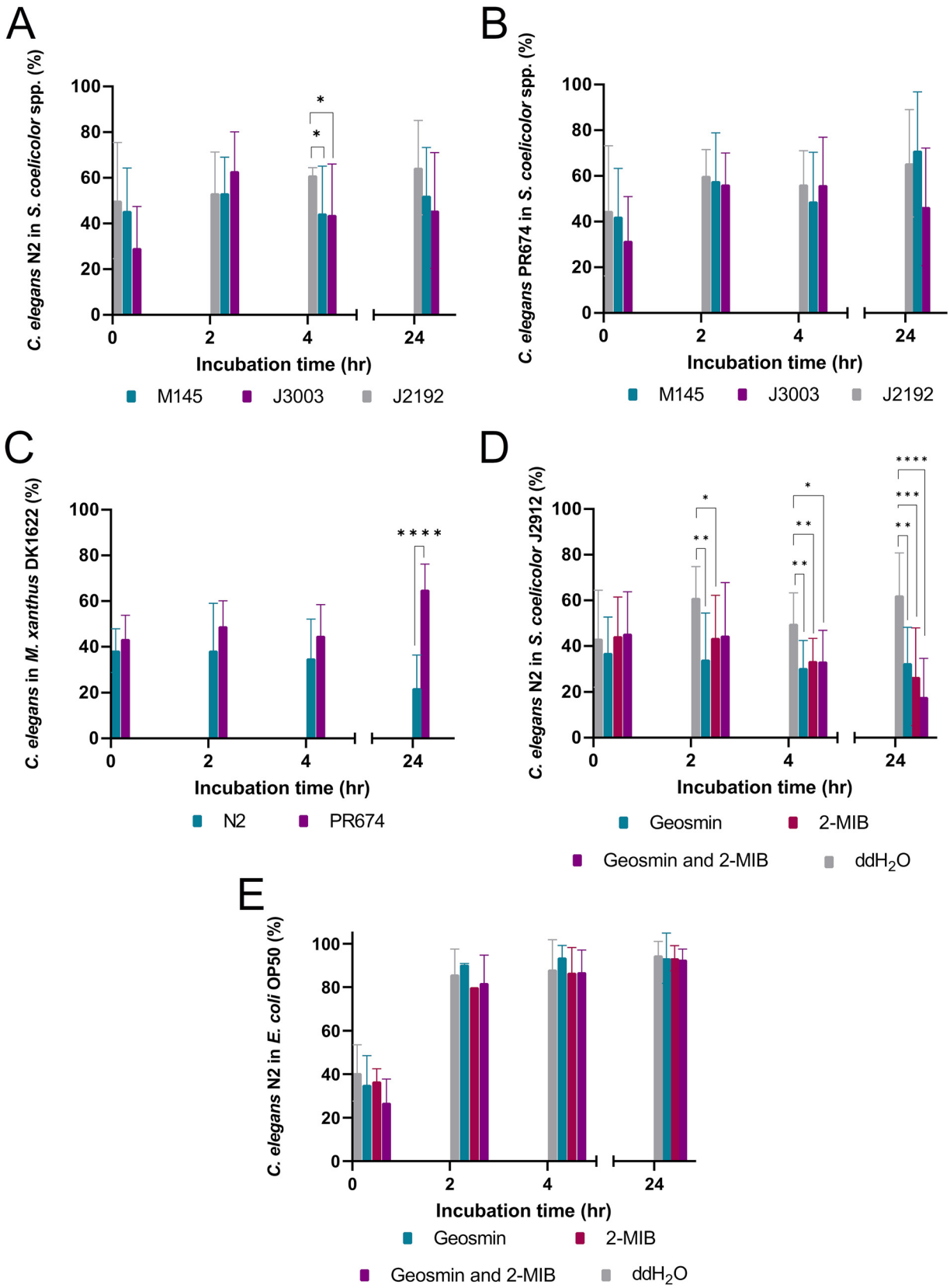

FIG 3 Colocalization of *C. elegans* and either *S. coelicolor* or *M. xanthus*. (A) Proportions of adult *C. elegans* N2 worms in colonies of *S. coelicolor* M145 (WT), J3003 (Δ*geoA*), and J2192 (Δ*geoA* Δ*mibAB*) as a function of time. (B) Proportions of *C. elegans* PR674 [*che-1*(*p674*)] (ASE-deficient) worms in colonies of *S. coelicolor* M145 (wild type), J3003 (Δ*geoA*), and J2192 (Δ*geoA* Δ*mibAB*) as a function of time.

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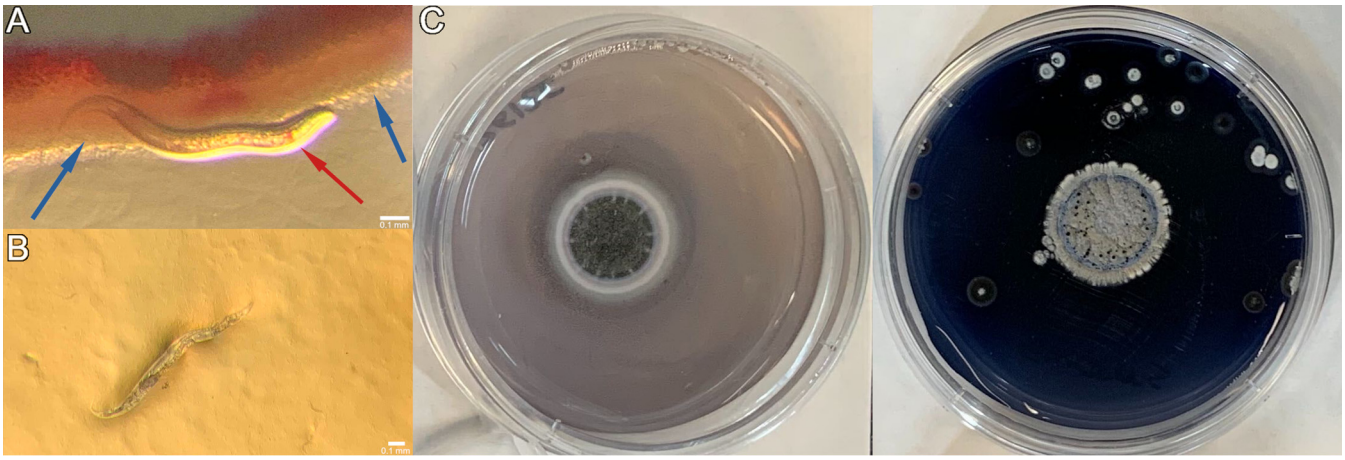

FIG 4 Visible effects of nematodal and bacterial interactions. (A) Consumption of *S. coelicolor* by *C. elegans*. Blue arrows indicate the bacterial colony, and the red arrow indicates the presence of bacteria in the *C. elegans* pharynx. (B) *C. elegans* on an *M. xanthus* lawn. The worm appeared to be digested by the bacteria over time. (C) *S. coelicolor* colony morphology in the absence (left) or presence (right) of *C. elegans*. Spores give the colonies a white color, while actinorhodin is an intense blue. Images show 10-day-old cultures at room temperature. Images were obtained in the course of conducting the predation assays described in the legend of Fig. 3 and are representative of the features that they depict.

(6, 7, 11). Here, we find that geosmin is sensed by the bacteriophagous nematode *C. elegans* and that it reduces interactions between the worms and *S. coelicolor*.

In the presence of geosmin, *C. elegans* switches its movement to one characterized by frequent changes in direction and high speed, an effect not seen in worms lacking functional ASE gustatory neurons (Table 1; see also Movies S1 to S4 in the supplemental material). Worms encountering geosmin-containing dry droplets frequently reversed their movement, similar to previously reported interactions with the *Streptomyces* metabolite dodecanoate (41), but this change in movement did not occur in the presence of the chemoattractant lysine (42). In predation assays, more worms were observed in *S. coelicolor* colonies deficient in both geosmin and 2-MIB production than in wild-type colonies, while worms lacking differentiated ASE neurons fed readily on both the *S. coelicolor* strains and wild-type *M. xanthus* (Fig. 3A to C). The direct addition of geosmin and/or 2-MIB significantly reduced grazing on *S. coelicolor* J2192 by *C. elegans* (Fig. 3D) but did not impact grazing on the favored prey *E. coli* OP50 (Fig. 3E). Grazing on *S. coelicolor* but not *E. coli* is harmful to *C. elegans* (46), suggesting that the avoidance of geosmin producers may require a separate measurement of food quality or is perhaps governed by a combination of chemoattractant and chemorepellent signals. Further studies will be necessary to determine the precise mechanism behind the aversive response that we have observed.

The function of geosmin as a warning signal is consistent with its reported effects on other eukaryotes and its prevalence across a range of unrelated microbes. Geosmin attracts the ant *Solenopsis invicta* because the terpene reliably indicates the presence of *Streptomyces* spp., and the toxic metabolites produced by these bacteria protect ant colonies from fungal infections (15). Similarly, geosmin discourages egg laying by *Drosophila*, whose young are susceptible to bacterial toxins (14), while signaling the presence of edible cyanobacteria and, thus, favorable breeding grounds to the more toxin-resistant mosquito *Aedes aegypti* (9, 47). While 2-MIB is less well understood, in our predation assays, it appears to function similarly to geosmin (Fig. 3D and E). 2-MIB

FIG 3 Legend (Continued)
(C) Proportions of *C. elegans* N2 and PR674 worms in colonies of *M. xanthus* DK1622 as a function of time. (D) Proportions of *C. elegans* N2 worms in colonies of *S. coelicolor* J2912 that were pretreated with 2.25 μg/mL geosmin, 2-methylisoborneol (2-MIB), or distilled, deionized water as a function of time. (E) Proportions of *C. elegans* N2 worms in colonies of *E. coli* OP50 that were pretreated with 2.25 μg/mL geosmin, 2-methylisoborneol (2-MIB), or distilled, deionized water as a function of time. Experiments in panels A to D were run nine times, with 10 adult hermaphrodites per study (n = 90). The experiment in panel E was run three times, with 10 adult hermaphrodites per study (n = 30). Worms were grown on *E. coli* OP50 and starved for 20 min prior to addition to the indicated organisms. Statistically significant deviations from J2192 (A and B), the wild type (C), or ddH_2O (D and E) are indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

biosynthetic genes are generally found in geosmin-producing actinobacteria (11), suggesting that the two terpenes either synergize or are detected by a nonoverlapping spectrum of microbial predators.

In principle, the acquisition of geosmin synthase by any marginal or toxic prey microbe could recapitulate the aposematic phenotype that we have observed, favoring lateral gene transfer between evolutionarily unrelated species and the evolution of Müllerian mimics (48, 49). Other aposematic signals exhibit positive frequency-dependent selection (50), and the evolution of Müllerian mimics in bacteria could favor the further lateral gene transfer of geosmin synthase. The ubiquity of geosmin in soil environments ensures that few predators are naive to the signal, while those that ignore it likely experience consistent fitness penalties from preying on toxic geosmin producers.

In conclusion, geosmin is detected by the predatory nematode *C. elegans* through interactions mediated by the ASE taste neurons. Geosmin is nontoxic to this organism, but nematodes are repelled by geosmin and strongly avoid geosmin-producing bacteria. When geosmin production was eliminated or *C. elegans* lacked functional ASE gustatory neurons, the worms became coated in bacterial spores and ingested toxic secondary metabolites, causing them to show signs of stress and become immobile. Concurrently, bacterial fitness declined through nematode feeding and the conversion of vegetative cells to metabolically inactive spores. Geosmin thus acts as an aposematic signal, honestly and reliably advertising the unpalatability of its producers and providing a mutual benefit to predator and prey. Geosmin is the first warning chemical to be identified in bacteria to date, and it not only shapes bacterial predator-prey interactions but also appears to mediate interactions between eukaryotes and bacteria across the globe.

MATERIALS AND METHODS

General information. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Inova 500 spectrometer. The δ values were referenced to $CDCl_3$ (^1H = 7.26 ppm; ^{13}C = 77.16). High-resolution mass spectrometry (HRMS) spectra were recorded on an Agilent Technologies 6220 oaTOF instrument by electrospray ionization (ESI). Mass spectrometry was performed on a 7 Tesla Thermo-Finnigan LTQ-FT mass spectrometer (Thermo Electron Corporation, San Jose, CA) fitted with an Ion Max electrospray source. The sample was analyzed by direct injection at 10 μL/min. The source voltage was 4.3 kV. Spectra were acquired in positive mode from *m/z* 50 to 300 at a resolution of 100,000 at *m/z* 200.

Chemicals. (i) General. The (±)-geosmin standard was obtained from Sigma-Aldrich, and (−)-geosmin was purchased from Fujifilm Wako Chemicals. Bovine serum albumin, levamisole, (1*R*)-(+)-camphor, and a 3.0 M methylmagnesium bromide solution in diethyl ether (catalog number 189898) were obtained from Sigma-Aldrich. FTC-casein and trypsin proteins were both obtained from Thermo Fisher Scientific. Methanol, ethanol, ethyl acetate (EtOAc), tetrahydrofuran (THF), hexane, and 2-butanone were acquired from ACS Chemicals and Fisher Scientific. 2-Methylisoborneol was synthesized in-house, as detailed below.

(ii) Media and buffers. The 1% CTT media (1% Casitone, 10 mM Tris-HCl [pH 7.6], 8 mM $MgSO_4$, 1 mM KH_2PO_4) was prepared from scratch. Luria-Bertani (LB) medium (10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract) was prepared from a premix, which was purchased from Bio Basic. Tryptic soy broth (TSB) (17 g/L casein peptone, 3 g/L soya peptone, 5 g/L NaCl, 2.5 g/L K_2HPO_4, 2.5 g/L glucose [pH 7.3]) premix was purchased from Sigma-Aldrich. Nematode growth medium (NGM) (3 g of NaCl, 2.5 g of peptone, 20 g of agar, 1 mL of 5-mg/mL cholesterol in ethanol, 1 mL of 1 M $MgSO_4$, 25 mL of 1 M [pH 6.0] KPO_4 in 1 L H_2O) was made from scratch. Worm M9 buffer (3 g/L KH_2PO_4, 6 g/L Na_2HPO_4, 5 g/L NaCl) was made from scratch. The Tris-buffered saline (TBS) buffer used in the protease assays was made from scratch (25 mM Tris, 0.15 M NaCl [pH 7.2]). The sodium phosphate buffer used in the CD assays was also prepared from scratch (pH 7.0; ionic strength, 0.014 M).

Strains and cultivation. (i) Bacteria. *Klebsiella aerogenes* ATCC 13048 was acquired from the American Type Culture Collection (ATCC). *Escherichia coli* MG1655 and *Burkholderia thailandensis* E 264 were a gift from Eric Déziel, INRS-IAF. *Myxococcus xanthus* DK1622, *Micrococcus luteus* DSM 20030, and *Bacillus subtilis* DSM 10 were obtained from the Leibniz Institute DSMZ (German Collection of Microorganisms and Cell Cultures). *Streptomyces coelicolor* M145, J3003, and J2192 originated from the John Innes Centre, Norwich, United Kingdom, and were gifts from Klas Flärdh. *Escherichia coli* OP50 was obtained from the Caenorhabditis Genetics Center (CGC).

M. luteus, *B. subtilis*, *B. thailandensis*, *K. aerogenes*, and *E. coli* were grown in LB medium at 30°C (37°C for *E. coli*) with rotation at 225 rpm for liquid cultures. Bacterial isolates were streaked onto 1.5% agar LB plates and placed at 30°C for 24 h (37°C for 16 h for *E. coli*) prior to experiments. *M. xanthus* DK1622 was grown in 1% CTT at 30°C at 225 rpm. Bacterial isolates were streaked onto 1.5% agar CTT plates and placed at 30°C for 3 days prior to experiments. *Streptomyces coelicolor* M145, J3003, and J2192 were grown at 30°C in TSB with rotation at 225 rpm, and isolates were streaked onto 1.5% agar tryptic soy

agar (TSA). Growth curves for *M. xanthus* DK1622 were generated by performing daily optical density at 600 nm (OD_{600}) measurements using a Varian Cary 100 Bio UV-visible (UV-vis) spectrophotometer.

(ii) *Caenorhabditis elegans*. The *C. elegans* strains were maintained on NGM plates with *E. coli* OP50 at 20°C according to a standard protocol (51). Wild-type N2 and mutant strains BR5514 [*tax-2* (*p671*); *tax-4*(*p678*)], CE1258 [*eat-16*(*ep273*)], CX2065 [*odr-1*(*n1936*)], CX2205 [*odr-3*(*n2150*)], CX5893 {*kyIs140*[*str-2*::*GFP* + *lin-15*(+)]*I*; *ceh-36*(*ky646*)}, NL2105 [*gpa-3*(*pk35*) *odr-3*(*n1605*)], and PR674 [*che-1* (*p674*)] were obtained from the CGC. Gravid nematodes were age synchronized and cleaned from bacterial and fungal contaminants using a bleaching mixture (2.5% NaClO, 0.5 M NaOH) before each experiment, as previously described (51). All *C. elegans* experiments were conducted using a standard stereomicroscope.

Geosmin quantitation. A culture of *M. xanthus* DK1622 in stationary phase was diluted to an OD_{600} of 0.125 and then diluted 1:100 in fresh 1% CTT. Samples were left shaking at 30°C at 225 rpm. Aliquots were drawn every 12 to 24 h until day 9. OD_{600} measurements were made on a Varian Cary 100 Bio UV-vis spectrophotometer. Samples over an OD_{600} of 1.0 were diluted in fresh 1% CTT medium to the range of 0.010 to 0.99. After 7 days of incubation, clumping was observed, and prior to measurements, cells were dispersed via passage through a serological pipette. Geosmin extractions were performed using a 1:1 ethyl acetate-water extraction with an *M. xanthus* DK1622 bacterial culture. Ethyl acetate samples were sonicated and then centrifuged for 1 min at 3,000 $\times$ *g* to collect the clean supernatant before injection for quantification by gas chromatography-mass spectrometry (GC-MS). To quantify medium versus cytoplasmic geosmin, the bacterial culture was centrifuged at 3,000 $\times$ *g* for 2 min. The supernatant was used to measure extracellular geosmin as detailed above, while the intracellular/cytoplasmic geosmin concentration was measured by exposing the pellet of *M. xanthus* cells to ethyl acetate, vortexing for 1 min, and then sonicating and centrifuging down for 1 min at 3,000 $\times$ *g*. The GC-MS system (7890B GC instrument coupled to a 5977B MS instrument; Agilent Technologies) was equipped with an autosampler and a split/splitless inlet kept at 300°C. Splitless injections (1.0 to 3.0 μL) were made on a 60-m DB-EUPAH column (0.25-mm internal diameter [ID] by 25-μm film thickness; Agilent Technologies) with the oven kept isothermal (80°C) for 8 min, ramped to 300°C at 15°C/min, and then held at that temperature for 5 min. The inlet was kept at 300°C throughout. The helium flow rate was 1.2 mL/min, with an He septum purge flow of 5 mL/min. Seven-level external calibration curves between 0.01 and 1.00 mg/L were used for quantitation.

Geosmin MIC. According to CLSI guidelines for direct colony suspension testing (52), bacterial cultures were transferred to LB broth and adjusted to a final turbidity equivalent to a 0.5 McFarland standard (1.5×10^8 CFU/mL). Bacteria were then mixed 1:1 with (±)-geosmin in 96-well plates to a final volume of 100 μL and then incubated at 30°C for 20 to 24 h. The effect of methanol alone was also evaluated, alongside growth and sterility controls for each strain. The MIC was defined as the concentration sufficient to inhibit bacterial growth as evaluated by the naked eye. After initial tests at concentrations similar to the level produced by *M. xanthus* DK1622 failed to inhibit growth, the quantity was increased, with a final testing range spanning from 1.0 to 500 mg/L.

Effect of geosmin on BSA thermal denaturation. CD analyses were performed using a Jasco J-715 spectropolarimeter. The cuvette width was 0.2 cm. The BSA concentration was 1.25 μM for all experimental and control assays. Fresh protein samples were prepared before each experiment and kept on ice. The (−)-geosmin concentration was 0.18 mM for the experimental assay, and the SDS concentration was 0.75 mM. All samples were diluted in phosphate-buffered saline (PBS) (53, 54). Subtractions and smoothing were done using Jasco J-715 software using controls of each chemical in PBS exclusively. Elevated temperature analysis was done at 80°C. The wavelength window measured was from 200 to 240 nm. Five scans were done for each measurement. Experiments were run in triplicate before data analysis using the Jasco J-715 instrument. Varying temperature analysis was performed at a wavelength of 208.6 nm and a heating/cooling rate of 1°C/min.

Protease activity assay. A Pierce fluorescent protease assay kit was purchased from Thermo Scientific. The kit contained FTC-casein, an *n*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin standard, and a TBS buffer pack (55).

According to the product sheet instructions (55), FTC-casein was diluted to a 5-mg/mL stock solution with ultrapure water. One 20-μL aliquot of the stock solution was diluted 1:500 in TBS to a final volume of 10 mL and a final concentration of 0.01 mg/mL. A 20-μL aliquot of trypsin stock solution (TSS) of 50 mg/mL was diluted to 1 mg/mL in TBS. To obtain the extracellular proteases of *M. xanthus* DK1622 without geosmin, the supernatant of a 10-day-old culture was extracted with EtOAc, and the organic layer was discarded. A 50% protease solution in TBS buffer was used and was kept on ice with FTC-casein and trypsin. Each fluorometric assay was measured using a Varian Cary Eclipse fluorescence spectrophotometer at an excitation wavelength ($\lambda_{excitation}$) of 494 nm and an emission wavelength ($\lambda_{emission}$) of 521 nm. The experiments were performed in the presence and absence of (±)-geosmin (1 mg/L) to evaluate the effect of (±)-geosmin on the activity of the proteases. Methanol was used as a negative control.

Chemotaxis assay. Geosmin chemotaxis experiments were assessed according to the protocol described previously by Bargmann et al. (56). On an NGM plate, 1 μL of (−)-geosmin at the indicated concentration was placed 1 cm from one end of the plate, and on the other symmetrical opposite end, 1 μL of Nanopure water was added as a control. One microliter of 1 M levamisole was added to each drop to prevent worm movement out of the drops, before drying the plates for 20 to 30 min. Pure 2-butanone was used as a positive control (57). Adult N2 hermaphrodites were washed 3 times in worm M9 buffer before addition to the center of the plates ($n = 50$). The plates were then incubated at room temperature (RT) for 1 h, at which point the number of worms in each spot was measured and the chemotaxis index was calculated.

Dry-drop avoidance assay. Avoidance assays were adapted from the amphid (head) avoidance assay described previously by Tran and coworkers (41). Age-synchronized N2 adult hermaphrodites were washed 3 times in worm M9 buffer and dried on clean NGM plates for 1 h inside a biosafety cabinet. Using a capillary tube, a drop of distilled, deionized water (ddH_2O) (control); 2.25 μg/mL (−)-geosmin; 43.3 μg/mL SDS; and/or 58.4 μg/mL lysine (all experimental samples were dissolved in ddH_2O) was placed a few millimeters ahead of a forward-moving worm and allowed to dry. If a worm initiated backward movement after contact with the dry drop, this was quantified as a response. If the worm continued a forward movement after contact with the dry drop, this was marked as a failure to respond. Each experiment was conducted with 40 nematodes ($n = 40$).

Behavioral assay. NGM agar with and without 0.54 μg/mL (−)-geosmin was poured into 24-well plates. Three adult hermaphrodites were added to each well and were videotaped for 10 min using a system that uses NIS Imaging BR version 3 software hooked up to a DSFi1c camera on a Nikon SMZ1500 microscope. The track line percentage [TL% = (D2S/Len) × 100, where D2S is the distance from the first to the current point and Len is the length from the first to the current point] was calculated using Imaris 9.5 (58), and data were processed with a custom-built python script (see Appendix S1 in the supplemental material). Briefly, the script read the .xls files produced by Imaris particle tracking analysis, split the data into five 2-min segments, and then extracted results for the track length and displacement of each worm for track line percentage determination. Videos were analyzed using WormLab software 2020.1.1 (59) to generate the peristaltic speed (micrometers per second) and the head movement periodicity (micrometers) of the worms. Peristaltic speed is defined as peristaltic track length, the length of the track made by the worm during its movement, divided over time. Head movement periodicity is the wavelength of the sinusoidal wave created by tracing the head of the worms as they crawl. Worms that lodged into crevices and ceased moving were removed prior to analysis. Data were processed using Microsoft Excel 2011. Experiments were conducted in triplicate.

Predation assay. Forty microliters of *S. coelicolor* (OD_{600} = 0.4 to 0.6), *M. xanthus* (OD_{600} = 0.2 to 0.3) was added to the center of a 5-cm-diameter NGM plate. The liquid was allowed to dry, and the plates were then incubated at 30°C (7 days for *S. coelicolor* and 3 days for *M. xanthus*). Ten adult hermaphrodites of the indicated strain were then added 2 to 5 mm away from the bacterial colony. Quantification of the worms inside and outside the bacteria was made at 0, 2, 4, and 24 h. Experiments were conducted nine times.

Terpene add-in assay. Add-in experiments were performed using *S. coelicolor* J2192 or *E. coli*, according to the same procedure as the one described above for the predation assays, with the following modifications. One hour prior to the addition of *C. elegans*, 40 μL of (−)-geosmin (2.25 μg/mL), (−)-2-methylisoborneol (40 μg/mL), or both was added to the bacterial colony and allowed to dry. Autoclaved Nanopure H_2O was used as a negative control. Each condition was evaluated with 10 adult N2 hermaphrodites, nine times for *S. coelicolor* ($n = 90$) or three times for *E. coli* (n = 30). Quantification was performed as described above for the predation assay.

***C. elegans* stress assay.** Solutions of (−)-geosmin (0.54, 5.4, and 54 μg/mL) and 2-methylisoborneol (1.5, 15, and 150 μg/mL) in LB broth were added to 24-well plates containing either NGM or NGM with *E. coli* OP50 according to methods described previously by H. Xiong et al. (60). LB broth was used as a negative control, and a sodium hypochlorite solution (2.625 mg/mL) in LB broth was used as a positive control. The plates were incubated overnight at RT. Age-synchronized plates of adult N2 worms were then washed in worm M9 buffer, dried on NGM plates, and then added to each well ($n = 5$). A lack of response to touch stimuli was used to infer stress and poor health. The number of unhealthy *C. elegans* worms was quantified at 0, 2, 4, and 24 h. Experiments were run in triplicate.

Synthesis of 2-methylisoborneol. Methylmagnesium bromide (4.95 mmol; 3 M) was added to a solution of (1*R*)-(+)-camphor (3.3 mmol) in anhydrous tetrahydrofuran (0.2 M) at 0°C. The reaction mixture was then heated to reflux for 10 h before being quenched with a saturated solution of ammonium chloride in water. The THF was removed *in vacuo*, and the remaining solution was extracted three times with EtOAc. The organic layers were then pooled, extracted with brine, and dried with sodium sulfate. The organic solvent was then removed *in vacuo* to give a white solid (325.8 mg). The crude mixture was then purified by flash chromatography (12% EtOAc in hexanes) to give the title compound as a white solid (48.7 mg, 8.77% yield, and >95% purity by ^{1}H NMR). ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.07 (dt, 1H, $J = 3.72$ Hz, 13.08 Hz), 1.70 (m, 2H), 1.39 (m, 4H), 1.24 (s, 3H), 1.10 (s, 3H), 0.87 (s, 3H), 0.85 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 79.6, 51.9, 48.9, 47.3, 45.4, 31.3, 27.0, 26.8, 21.4, 21.2, 9.9. Calculated molecular weight (MW) for $C_{11}H_{20}O$: 168.1514. Found MW at high resolution (ESI-MS): 151.1480 $[M - H_2O + H]^+$.

Statistical analysis. Unless otherwise noted, all statistical analyses were performed using Student's *t* test calculations with a two-tailed distribution and unequal variance. Statistical significance at a *P* value of <0.05 is noted in the figures (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$). All replicates are distinct samples (biological replicates). Data were visualized with GraphPad Prism 9.0. For the dry-drop avoidance assay, statistical analyses were performed using independent-sample Z tests. All samples were compared to each other, with a Benjamini-Hochberg procedure applied to correct for potential false positives ($\alpha = 0.25$). All comparisons except for 0.15 mM SDS versus 2.25 μg/mL of geosmin were statistically significant.

Data availability. All data described in the manuscript is available in the main text or the supplemental material.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.1 MB.

SUPPLEMENTAL FILE 2, MP4 file, 7.1 MB.

SUPPLEMENTAL FILE 3, MP4 file, 9.8 MB.

SUPPLEMENTAL FILE 4, MP4 file, 0.3 MB.

SUPPLEMENTAL FILE 5, MP4 file, 0.3 MB.

SUPPLEMENTAL FILE 6, MP4 file, 0.3 MB.

ACKNOWLEDGMENTS

We thank P. Prevost and the Forgione lab for assistance with the synthesis of 2-methylisoborneol; K. Flärdh for supplying *S. coelicolor* M145, J3003, and J2192; E. Despland for microscopy support; M. DiFalco for assistance with HRMS; and L.K. Freeman for discussions and feedback. Imaging support was provided by the Concordia Centre for Microscopy and Cellular Imaging, while CD spectroscopy and fluorimetry were performed with the assistance of the Concordia Integrated Platform for Biomolecular Function, Interactions and Structure.

This work was supported by funds from the National Sciences and Engineering Research Council (NSERC) and the Fonds de Recherche du Québec-Nature et Technologie (FRQNT). The Caenorhabditis Genetics Center is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440).

B.L.F. and L.Z. conceived the study. L.Z. performed all listed experiments with assistance from A.I. and Y.G. (GC-MS); I.O., K.M., and A.P. (*C. elegans*); and C.L. (microscopy). I.O. and A.P. helped perform the behavioral, predation (*S. coelicolor*), and terpene add-in assays. K.M. and A.P. aided with the chemotaxis assay. C.L. prepared the python script. B.L.F. and L.Z. prepared the manuscript, with feedback from all authors.

We declare no conflict of interest.

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